REMARKS

Rejection under 35 U.S.C. §112, second paragraph

The claims have been amended to clarify the methods and conditions under which the molecular weight analysis was performed. Support can be found in the specification, e.g., Page 15, lines 24-25; Page 29, lines 23-26; Page 34, lines 8-16; Page 38, lines 9-10. This amendment does not change the scope in any way, but merely clarifies, what would have been apparent upon reading the specification, how the molecular weight was determined.

The hybridization conditions disclosed in the specification were standard conditions accompanying the DIG system (Roche). See, Specification, Page 46 and Claim 124. "The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). Hybridization is conventional in the art. The Federal Circuit has held that a "patent need not teach, and preferably omits, what is well known in the art. " See, *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987).

The specification has been amended by inserting the conditions of the stringency washes referred to on Page 46. This amendment is not new matter. See, accompanying declaration. Claims 208 and 209 have also been added which recite these conditions.

Rejection under 35 U.S.C. §102

It is stated on Page 5 of the Office action dated January 28, 2005 that "Merks [WO 94/05703] isolated the [20-kDa] polypeptide via SDS gels, this results in a protein band which is isolated and free from other polypeptides which would migrate faster or slower."

Merks expressly refer to the disclosed protein as being only "partially purified." On Page 1, lines 2-6, Merk states: "The present invention involves a monoclonal antibody (Mab) with the specificity for a 20,000 dalton cell surface protein of Neisseria meningitidis, a cell line that produces said antibody, and the partially purified 20,000 dalton cell surface protein." (Underling added.) See, also Page 5, lines 5-6, where it is referred to as "significantly purified." Thus, the

examiner's characterization of it as being isolated and free from other proteins is not consistent with Merks's own view of her invention. This is adequate to rebut the rejection since no evidence has been presented in the Office action to establish that the 20-kDa SDS-PAGE band described by Merks inherently has the property of being isolated. To the contrary, Merks never makes this assertion and explicitly states the opposite.

Although further argumentation is unnecessary, it is further noted that the one-dimensional SDS-PAGE gels utilized in Merks to resolve the alleged 20-kDa protein would not have produced an isolated protein, nor a protein which is free of other N. meningitidis proteins. SDS-PAGE separates proteins based on molecular weight, and typically there will be a mixture of different proteins migrating at about the same molecular weight, e.g., proteins having about the same weight, but having different amino acid sequences and isoelectric points. In addition, proteolysis of larger proteins can occur during sample preparation, resulting in a range of proteolytic fragments, including fragments which could migrate to about 20-kDa. Thus, one-dimensional SDS-PAGE does not result in isolated proteins as alleged.

The attached publication by Bernardini et al. (2004) [Exhibit 1] establishes that there are numerous proteins that migrate in the molecular weight range of about 20-kDa, and thus would not have been separated by one-dimensional gel electrophoresis. Bernardini et al. performed a proteome analysis of Neisseria meningitidis using two-dimensional gel electrophoresis. Table 1 shows the following proteins identified in the publication:

Spot	Exp. Mw (Da)
(94)	17,539
(158)	17,994
(51)	18,940
(255)	19,349
(273)	19,432
(221)	19,767
(224)	20,629
(223)	20,717
(95)	21,621
(222)	21,714
(156)	22,468
(225)	22,953
(300)	23,659
(97)	24,682

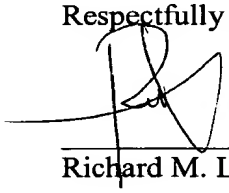
These results expressly demonstrate the presence of multiple species in the range reported by Merks. Although Merks utilized an extraction procedure to enrich for outer membranes (e.g., Merks, Page 6, line 15; Page 10, lines 15-16), these were prepared from cell lysates and would not likely have been free of other cellular proteins. Moreover, at least one of these proteins – Spot 221 – which is closest is molecular weight to the 20-kDa protein, is described as a pilus assembly protein and a cell envelope structure. See, Bernardini, Page 2910. Given the resolving power and sensitivity of one-dimensional SDS-PAGE, it is evident that multiple protein species (e.g., at least the additional Spot 221) would have been present in the 20-kDa protein allegedly obtained by Merks, thus consistent with Merks own characterization of it as being only "partially purified."

For these reasons, the claims are not anticipated by Merks, and the rejection should be withdrawn.

If there are any remaining issues which could be expedited by a telephone conference, the Examiner is courteously invited to telephone counsel at the number indicated below.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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Proteome analysis of *Neisseria meningitidis* serogroup A

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Neisseria meningitidis is an encapsulated Gram-negative bacterium responsible for significant morbidity and mortality worldwide. Meningococci are opportunistic pathogens, carried in the nasopharynx of approximately 10% of asymptomatic adults. Occasionally they enter the bloodstream to cause septicaemia and meningitis. Meningococci are classified into serogroups on the basis of polysaccharide capsule diversity, and serogroup A strains have caused major epidemics mainly in the developing world. Here we describe a two-dimensional gel electrophoresis protein map of the serogroup A strain Z4970, a clinical isolate classified as ancestral to several pandemic waves. To our knowledge this is the first systematically annotated proteomic map for *N. meningitidis*. Total protein samples from bacteria grown on GC-agar were electrophoretically separated and protein species were identified by matrix-assisted laser desorption/ionization time of flight spectrometry. We identified the products of 273 genes, covering several functional classes, including 94 proteins so far considered as hypothetical. We also describe several protein species encoded by genes reported by DNA microarray studies as being regulated in physiological conditions which are relevant to natural meningococcal pathogenicity. Since menA differs from other serogroups by having a fairly stable clonal population structure (*i.e.* with a low degree of variability), we envisaged comparative mapping as a useful tool for microevolution studies, in conjunction with established genotyping methods. As a proof of principle, we performed a comparative analysis on the B subunit of the meningococcal transferrin receptor, a vaccine candidate encoded by the *tbpB* gene, and a known marker of population diversity in meningococci. The results show that TbpB spot pattern variation observed in the maps of nine clinical isolates from diverse epidemic spreads, fits previous analyses based on allelic variations of the *tbpB* gene.

Keywords: Genocloud / Matrix-assisted laser desorption/ionization mass spectrometry / Meningococcus / Two-dimensional gel electrophoresis

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1 Introduction

Neisseria meningitidis (meningococcus) is a Gram-negative bacterium that exclusively infects humans and colonizes the nasopharynx. Colonization of mucosal membranes can result in an asymptomatic carriage or, occasionally, in a highly invasive infection causing life-threatening disease. *N. meningitidis** is a leading cause of septicaemia and meningitis in the world with a few hundred thousands cases estimated *per* year, predominantly in young children and teenagers [1, 2]. When *N. meningitidis* occasionally crosses the nasopharyngeal epithelium and enters the bloodstream, it can replicate very rapidly in the blood, spread to the whole organism and cause septicaemia; furthermore, it can interact with brain microvessel endothelial cells, cross the blood-brain barrier and even-

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* The complete annotated map will be submitted to the EBP Berlin database at http://www.mplib-berlin.mpg.de/

tually cause meningitis [3, 4]. Meningococcal sepsis, or meningococcemia, occurs in only 5 to 20% of patients, and is characterized by an abrupt onset of fever and a petechial or purpuric rash, which may progress to purpura fulminans. Septicaemia is often associated with a rapid onset of hypotension, acute adrenal hemorrhage and multi-organ failure. Despite treatment with appropriate antimicrobial agents, the overall case fatality rates have remained relatively stable at 9 to 12%, with a rate of up to 40% among patients with Meningococcal sepsis [5].

N. meningitidis isolates are classified into 12 serogroups based on specific immunochemical features of the polysaccharide capsules [6]. Five of these serogroups, namely A, B, C, Y and W135, account for virtually all isolates which cause meningococcal disease. The frequency of disease differs with the geographical area, ranging from annual endemic levels to epidemic levels. While endemic infections (Europe and Americas) are usually caused by meningococci belonging to serogroups B and C (menB and menC), most of the epidemics/pandemics (Africa and China) are caused by serogroup A (menA) strains. Interestingly, endemic *N. meningitidis* isolates are rather diverse and without clonal population features, whereas epidemic causing meningococci belong to fairly uniform clonal groupings, descending from common ancestors [7].

Although partially annotated incomplete proteome maps have been previously published for the serogroup B MC58 strain [8] and a serogroup C strain [9], this paper presents, to the best of our knowledge, the first systematic proteome mapping of a *N. meningitidis* clinical isolate and establishes the basis for a comprehensive meningococcal proteome database. For this work we selected the serogroup A strain Z4970 for two main reasons: (i) as mentioned above, serogroup A meningococci have a clonal population structure, and therefore a serogroup A reference map is expected to be more easily comparable to maps of other epidemiological isolates, thereby facilitating future comparative proteomic studies; (ii) strain Z4970 is a clinical isolate described as ancestral to other strains responsible for several subsequent epidemics [10]. As a test of the potential use of the annotated 2-DE map data for the study of menA microevolution and epidemiology, we also report a preliminary comparative analysis performed on the B subunit of the meningococcal transferrin receptor, a potential vaccine candidate encoded by the *tbp*B gene which has already been described as a useful marker of population diversity for meningococci. The results show that the spot patterns observed in the 2-DE maps of nine clinical isolates belonging to diverse epidemic spreads, are consistent with published analyses based on gene sequence typing which included the allelic variants of the *tbp*B gene [10].

2 Materials and methods

2.1 *N. meningitidis* strain and whole-cell extract preparation

N. meningitidis serogroup A bacteria were grown to confluence on GC gonococcal agar plates (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 4 g/L glucose, 0.1 g/L glutamine, and 2.2 g/L co-carboxylase, at 37°C in a humidified atmosphere containing 5% CO_2. Bacteria were harvested from three plates, washed twice in 10 mL of PBS containing an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) and centrifuged at 8000 × *g*, for 10 min at 5°C. The pellets were resuspended in 1 mL of reswelling buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v amidosulfobetaine-14 [11], 2% v/v Pharmalyte pH 3–10, 2 mM tributylphosphine, 1% w/v DTT). Benzoase (1000 U) and $MgSO_4$ (2 mM) were added and the reaction was incubated at room temperature for 30 min and then centrifuged at 12 000 × *g*, for 2 h, at 15°C. After centrifugation, the total protein concentration in the supernatant was evaluated by the Bradford method [12].

2.2 2-DE

Fifty μg (analytical) or 1 mg (preparative) protein samples were brought to a final volume of 700 μL with reswelling buffer and a trace of bromophenol blue. Proteins were adsorbed onto an Immobiline DryStrip (18 cm, pH 3–10 nonlinear gradient) for 6–18 h. IEF was carried out on a horizontal electrophoresis system Multiphor II (Amersham Biosciences, Uppsala, Sweden). The voltage was linearly increased from 300 to 3500 volts during the first 3 h and then stabilized at 5000 volts for 22 h (total 110 kVh). The IPG strips were then equilibrated in 6 M urea, 30% w/v glycerol, 2% w/v SDS, 0.05 M Tris-HCl pH 6.8, 2% w/v DTE and later also with 2.5% w/v iodoacetamide. Electrophoresis in the second dimension was carried out on a 9–16% polyacrylamide linear gradient gel (18 × 20 cm × 1.5 mm) with a constant current of 40 mA until the dye front reached the bottom of the gel. Analytical gels were stained with ammoniacal silver nitrate as previously described [13], while preparative gels were stained with colloidal Coomassie G-250 [14].

2.3 In-gel protein digestion

Spots from 2-DE were excised from the gel, triturated and washed with water. Proteins were in-gel reduced with 10 mM DTT in 100 mM NH_4HCO_3 (45 min, at 55°C) and *S*-alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 (30 min, at 25°C, in the dark). Gel particles were washed with 50 mM NH_4HCO_3 and ACN, dried and rehy-

drated with the digestion solution (12.5 ng/μL of trypsin in 50 mM NH_4HCO_3, 5 mM $CaCl_2$). After incubation for 1 h, at 5°C, supernatants were replaced by 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and gel particles were incubated overnight, at 37°C. Digest aliquots (1 mL) were withdrawn and directly analyzed by MALDI-TOF MS. Gel particles were eventually extracted with 1:1 v/v 25 mM NH_4HCO_3/ACN by sonication and peptide mixtures were concentrated. Samples were desalted using mZipTip C18 pipette tips (Millipore, Bedford, MA, USA) before MS analysis.

2.4 MALDI-TOF MS

Peptide mixtures were loaded on the MALDI target together with CHCA as matrix, using the dried droplet technique. Samples were analyzed with a Voyager-DE PRO spectrometer (Applied Biosystems, Framingham MA, USA). Peptide mass spectra were acquired in reflectron mode and data were elaborated using the Data Explorer 5.1 software provided by the manufacturer. Internal mass calibration was performed with peptides derived from trypsin autoproteolysis.

PSD fragment ion spectra were acquired after isolation of the appropriate precursor using timed ion selection. Fragment ions were refocused onto detector by stepping the voltage applied to the reflectron in the following ratios: 1.000 (precursor ion segment), 0.960, 0.750, 0.563, 0.422, 0.316, 0.237, 0.178, 0.133, 0.100, 0.075, 0.056 and 0.042 (fragment segments). Individual spectral segments were acquired in linear mode and were superimposed by using Data Explorer 5.1 software (Applied Biosystems). All precursor ion segments were acquired at low laser power (variable attenuator = 1950) for less than 200 laser pulses to avoid saturation of the detector. The laser power was increased 200 units for all remaining segments of the PSD acquisitions. Typically, 300 laser pulses were acquired for each fragment-ion segment. PSD data were acquired with an Acqiris digitizer at a digitization rate of 500 MHz.

2.5 Protein identification

The ProFound software package was used to identify spots from independent nonredundant sequence databases by PMF experiments [15]. Candidates with ProFound estimated Z scores > 2 were further evaluated by the comparison with M_r and *pI* experimental values obtained from 2-DE. The occurrence of protein mixtures was ascertained by sequential searches for additional protein components using unmatched peptide masses. The Protein Prospector software package was used to identify spots from independent nonredundant sequence databases using fragment ions obtained from PSD experiments [16].

2.6 *In silico* analysis of 2-DE maps

The digitalized images were obtained by scanning the gels with a Laser Densitometer (4000 × 5000 pixels; 121 bits/pixel; Molecular Dynamics, Sunnyvale, CA, USA) following qualitative and quantitative analysis by Melanie II 2D-PAGE and PDQuest softwares (Bio-Rad, Hercules, CA, USA). Spot intensity values were obtained in pixel units and normalized to the total absorbance of the gel. In order to evaluate experimental *pI* and M_r coordinates for each single spot, *N. meningitidis* samples were comigrated with a protein extract of *Saccharomyces cerevisiae* strain K310, whose proteomic 2-DE map is well characterized [17, 18]. Computation of theoretical *pI*/M_r and subcellular localization prediction for identified proteins were performed with the Compute *pI*/M_r tool at the ExPASy server (http://www.expasy.ch/tools/pi_tool.html) and PSORT-B program for Gram-negative bacterial sequences (http://www.psort.org/) [19]. A validation of *pI*/M_r calibration of our gels was obtained by comparing the distribution of theoretical and experimental values and confirmed by MS-derived identification which allowed additional reference spots to be used for more reliable gel matching.

3 Results and discussion

3.1 Protein separation, identification and quantification

A menA, whole-cell protein extract was separated on a 2-DE gel covering the pH 3–10 (IPG nonlinear gradient) and M_r 8–200 kDa (linear gradient) ranges. The gel was loaded with 1 mg of protein sample and stained with colloidal Coomassie blue. Using Melanie II 2-D PAGE analysis software, approximately 1220 protein spots were resolved, as shown in Fig. 1A. Gels loaded with 50 μg protein followed by silver staining were also produced. This type of gel (Fig. 1B) resolved 1518 spots. Reproducibility was assessed by comparing six replicas of the gels which were perfectly superimposable (data not shown). Proteins spots were annotated only if detectable in all gels. Fig. 2 shows four details of a Coomassie-stained 2-DE gel, where the identified proteins are indicated and numbered.

Spots from one single Coomassie-stained gel or from three silver-stained gels were excised and analyzed for protein identification by MALDI-TOF MS. A peptide mass fingerprint procedure was generally adopted. In some cases, a verification of the identified species was obtained by PSD analysis of one or more tryptic peptides. The peptide fingerprint and PSD spectra of peptides determining the identification of two putative proteins,

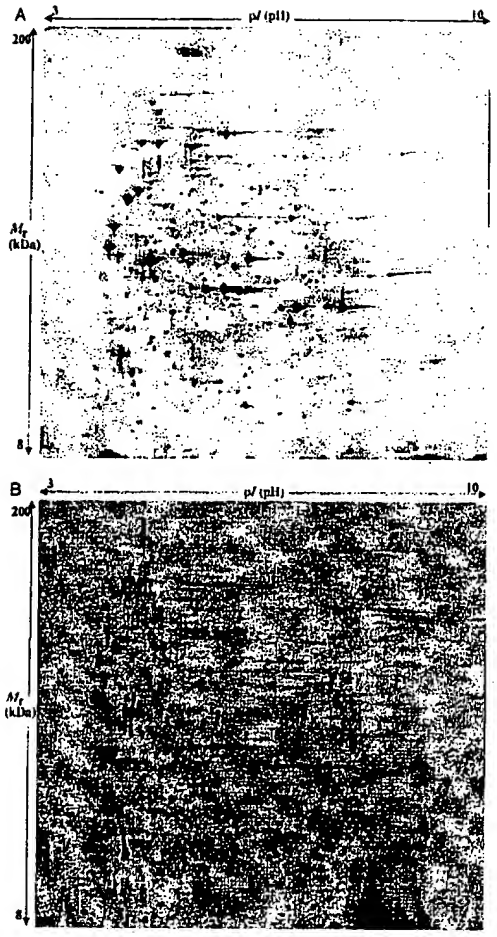
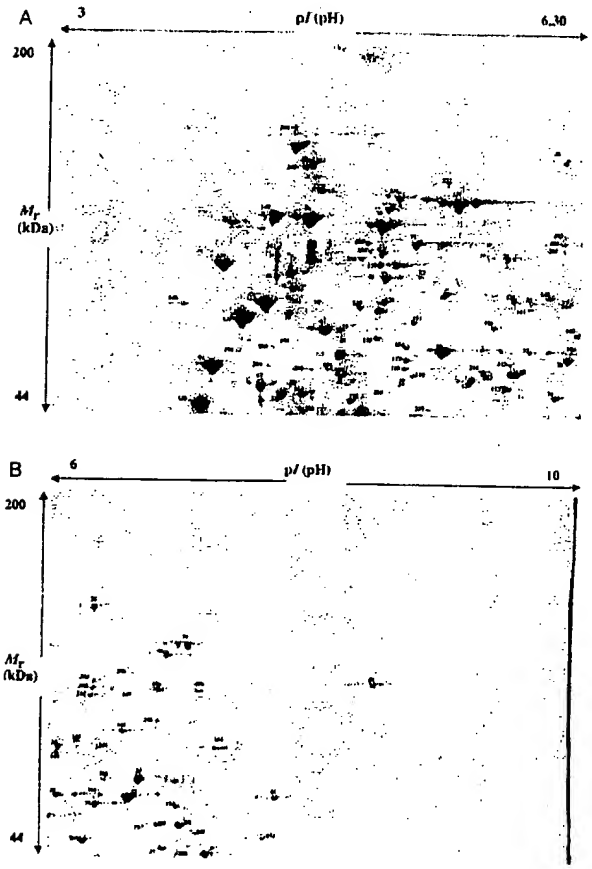


Figure 1. 2-DE map of a total protein extract from *N. meningitidis* serogroup A subgroup III strain 4970. (A) gel stained with colloidal Coomassie Blue G-250, (B) gel stained with silver nitrate.

namely putative cysteine synthase (spot 66) and putative polyamine permease substrate-binding protein (spot 104), are reported as examples (Fig. 3). Four spots (169a, 184, 195, 310) were found to contain overlapping proteins either in Coomassie- or silver-stained gels. The presence of multiple proteins within the same spot did not impair a reliable identification. Out of a total of 287 protein spots analyzed, 273 proteins were identified, which corresponds to approximately 13% of the 2121 ORFs predicted from the genome sequence [20]. A complete list of the identified proteins is reported in Table 1. The reliability of identification was evaluated by comparing the experi-

mentally determined p*I*/*M*$_r$ values of the proteins with the theoretical coordinates predicted from *N. meningitidis* translated gene sequences using the program TagIdent from the ExPASy server [21].

Of the proteins annotated in the map here reported, 18 proteins were classified in the Swiss-Prot database as hypothetical, 71 as putative, four as probable and one as possible. We thus establish in this work the expression of 94 proteins whose existence was not so far demonstrated. Table 2 lists the 20 proteins with the highest spot volume in the map, altogether accounting for around 30% of the total spot volume. Spots containing overlapping peptides were excluded from this evaluation. The presence among the most abundant proteins of elongation factors, outer membrane proteins and chaperones, as well as proteins generally involved in protein synthesis, was in good agreement with that reported for the proteomes of other bacterial pathogens [22]. The high recovery of elongation factor Tu is consistent not only with



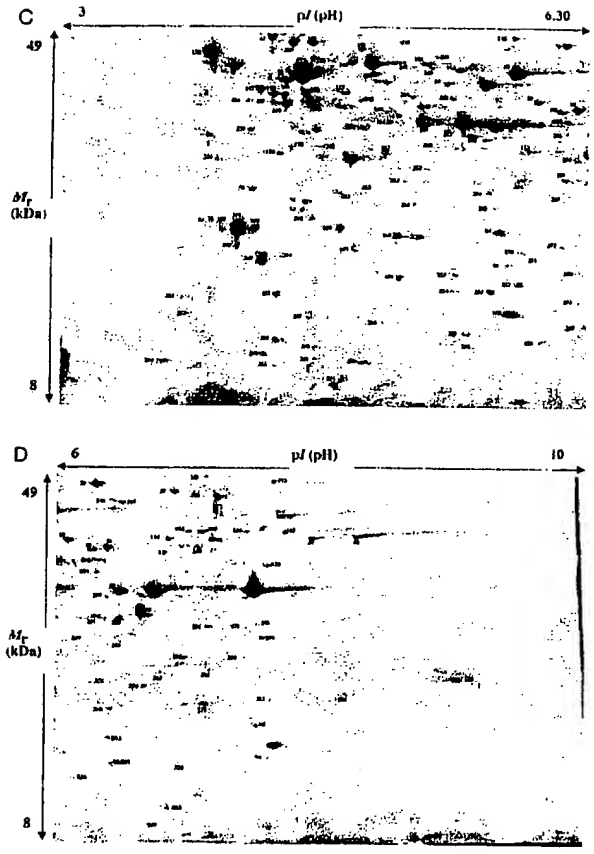


Figure 2. Four zoomed details (A, B, C, D) of the proteome map of strain Z4970 shown in Fig. 1. Spot numbering refers to Table 1 which reports protein identification by MS analysis.

its role in translation elongation, but also with its chaperone properties in protein folding, as found in *Escherichia coli* [23]. Moreover, in *N. meningitidis* enzymes necessary for amino acid biosynthesis and energy metabolism also seem to be expressed in quite high amounts. An *in silico* evaluation of the subcellular localizations of the most abundant protein species, shows that these include the cytoplasm, and the inner and outer membranes, thus indicating that the 2-DE map allowed to separate proteins having different hydrophilic/hydrophobic features to be separated. The predicted cellular localization was obtained by using the PSORT-B program [19] adopting the cut-off value of 7.5 suggested by the program itself. Subcellular localization was also obtained directly from Swiss-Prot entries. As reported in Table 1, when all the possibilities were equally probable the notation U (for unknown) was adopted.

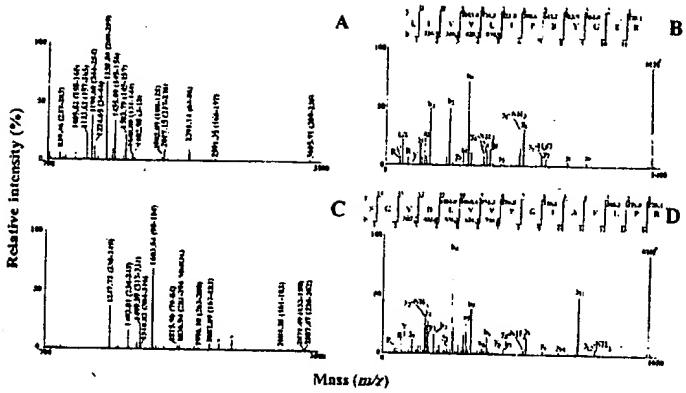


Figure 3. MALDI-TOF MS analysis of *N. meningitidis* protein spots 66 and 104. Peptide mass fingerprint spectra of tryptic digests from spots 666 and 104 are shown in (A) and (C), respectively. MALDI-TOF PSD mas spectra of the peptide with monoisotopic MH^+ signal at *m/z* 1358.64 (from A) and 1603.94 (from C) are shown in (B) and (D), respectively. Measured mass values and assigned fragment ions are indicated. The proteins were identified as cysteine synthase and polyamine permease substrate-binding protein, respectively.

3.2 M_r and pI of identified protein species

Identified proteins ranged from theoretical pI 4.55 (spot 146) to 9.04 (spot 189) and from theoretical M_r 156 kDa (spot 301) to 15 kDa (spot 258). M_r and pI values of protein spots were experimentally determined and compared with gene-deduced M_r/pI coordinates (Table 1). The theoretical M_r/pI distributions were predicted using the ExPASy server Tagident tool [21] on *N. meningitidis* proteins present in the Swiss-Prot/TrEMBL databases. The majority of gel estimated and theoretical M_r/pI values matched quite well (Fig. 4) at least within a window of M_r values between 20 and 60 kDa and pI values from 4.5 to 6.8. Interestingly, all the proteins showing the greater discrepancy between theoretical and experimental pI values are hypothetical or putative proteins and most of them were found to be more acidic than predicted.

When M_r differences were considered, both lower and higher experimental values respective to theoretical ones were observed. Besides the possibility of genomic annotation errors, lower M_r values could be due to post-translational processing or proteolysis, while higher M_r could result from PTM causing covalent binding of chemical groups to the amino acid backbone without significantly modifying the pI. Alternatively, a cause of discrepancy can be an anomalous electrophoretic mobility peculiar to a given protein. This is for instance the case for reduction modified protein Meningococcus (RmpM), alias class 4 outer membrane protein, which has a theoretical M_r of about 25 kDa or 23 kDa for the mature form without the *N*-terminal signal peptide, but displays an electrophoretic mobility yielding an apparent M_r of about 32 kDa in SDS-PAGE [24]. A similar observation was reported for the corresponding RmpG protein in gonococcus [25], and for the neuronal phosphoprotein DARPP-32 [26]. Glycosylations and lipidations are the most probable modifications causing the M_r changes observed. Lipidation is involved in PTM of dihydrolipoamide *S*-acetyltransferase (spot 88) and dihydrolipoamide dehydrogenase (spots 86 and 142) which are, together with pyruvate dehydrogenase (spot 127), functional parts of the outer membrane pyruvate dehydrogenase complex. They share the same *N*-terminal lipoyl-domain [27] which is probably also contained in the structure of putative dihydrolipoamide dehydrogenase (spots 69, 199). Lipid modification of prelipoproteins is a key biochemical pathway necessary to allow protein localization on the bacterial cell surface and is essential for growth and viability in *E. coli* and *Salmonella typhimurium* [28] or for virulence in *Streptococcus pneumoniae* [29].

Table 1. List of proteins identified in *N. meningitidis* serogroup A strain Z4970

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
1	Serine hydroxymethyl-trasferase	*glyA*	1254	Q9XAY7	C	Amino acids biosynthesis (serine family)	44 987	6.32	44 341	6.76	30
2	Putative citrate synthase	*gltA*	1148	Q9JQX0	C	Energy metabolism (tri-carboxylic acid cycle)	48 121	6.33	44 438	6.75	51
3	Putative citrate synthase	*gltA*	1148	Q9JQX0	C	Energy metabolism (tri-carboxylic acid cycle)	48 121	6.33	44 208	6.75	38
15	Glutamine synthetase	*glnA*	2128	Q9JSU6	C	Amino acids biosynthesis (glutamate family)	52 076	5.20	62 481	5.28	26
16	Hypothetical protein NMA1557		1557	Q9JU05	U*	Unknown	56 633	5.11	58 203	5.28	34
22	Glutaredoxin		1141	Q9JQS4	C	Detoxification	26 911	4.80	27 460	4.67	60
23	Glutaredoxin		1141	Q9JQS4	C	Detoxification	26 911	4.80	27 414	4.61	44
29	Phosphoribosylformyl-glycinamidine synthase	*purL*	0445	Q9JWC5	C	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	143 791	5.28	nd	4.99	29
30	ClpB protein	*clpB*	1683	Q9JTP9	U*	Degradation of macromolecules (proteins, peptides and glycopeptides)	95 093	5.45	84 015	5.39	42
31	Putative polyribonu-cleotide nucleo-tidyltransferase	*pnp*	0969	Q9JV72	C*	Degradation of macromolecules (RNA)	76 258	5.35	76 414	5.36	32
32	Putative maltose phosphorylase	*mapA*	2098	Q9JSW8	U*	Degradation (carbon compounds)	85 483	5.63	78 011	5.75	18
33	Dihydroxy-acid dehydratase	*ilvD*	1361	Q9JUE0	U(c)*	Amino acid biosynthesis (branched chain family)	66 857	5.78	69 316	6.09	27
34	Putative threonine synthase	*thrC*	1440	Q9JU91	U*	Amino acids biosynthesis (aspartate family)	51 765	5.64	50 349	5.52	41
35	Putative formate-tetra-hydrofolate ligase	*fhs*	0617	Q9JVY8	U(c)*	Central intermediary metabolism	59 122	5.85	59 047	6.47	32
36	Proline dehydrogenase	*putA*	2084	Q9JSY1	C*	Degradation of macro-molecules (proteins, peptides and glycopeptides)	138 326	6.36	nd	6.22	24
37	Possible periplasmic protein		0282	Q9JWN5	U*	Cell envelope (periplasmic proteins)	46 119	6.68	45 482	6.56	39
38	Methylcitrate synthase/citrate synthase 2	*prpC*	2054	Q9JRA5	U(c)*	Miscellaneous	42 818	6.61	42 811	6.85	39
39	Porin A	*porA*	1642	Q9JPT6	IN/OM	Cell envelope (membranes, lipoprotein and porins)	41 661	8.90	40 270	nd	66
40	Porin A	*porA*	1642	Q9JPT6	IN/OM	Cell envelope (membranes, lipoprotein and porins)	41 661	8.90	40 689	nd	77
46	Phosphoenolpyruvate carboxylase	*ppc*	0374	Q9JWH1	U(c)*	Energy metabolism (tri-carboxylic acid cycle)	101 071	6.26	nd	6.55	24
47a	Transferrin-binding protein B	*tbpB*	2025	O68937	U*	Pathogenicity	75 176	6.27	78 590	6.51	26

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
47b	Transferrin-binding protein B	*tbpB*	2025	068937	U*	Pathogenicity	75 176	6.27	78 590	6.72	20
50	Outer membrane protein class 4	*rmpM*	2105	P38367	IN/OM	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	26 140	6.53	32 129	6.42	67
51	Hypothetical protein NMA1094		1094	Q9JUX7	U(c)*	Conserved hypothetical protein	20 954	6.60	18 940	6.87	57
52	Glutamate dehydrogenase	*gdhA*	1964	Q9JT56	U*	Amino acids biosynthesis (glutamate family)	48 462	5.80	44 696	6.01	67
53	ATP synthase alpha chain	*atpA*	0517	Q9JW72	U(c)*	Energy metabolism (ATP-proton motive force)	55 318	5.57	57 588	5.77	35
54	Acetate kinase 1	*ackA1*	1718	Q9JTM0	C*	Energy metabolism (respiration)	42 467	5.67	42 146	5.92	75
55a	Acetate kinase 1	*ack1*	1718	Q9JTM0	C*	Energy metabolism (respiration)	42 467	5.67	42 477	5.76	50
55b	Cysteine desulfurase	*iscS*	1594	Q9JTX0	U(c)*	Miscellaneous	44 742	5.57	42 477	5.76	
56	Elongation factor G	*fusA*	0135	Q9JX07	C	Synthesis and modification of macromolecules (protein translation and modification)	77 215	5.08	81 713	5.05	60
57	Elongation factor Tu	*tuf*	0134	Q9JRI5	C	Synthesis and modification of macromolecules (protein translation and modification)	42 908	5.07	44 347	5.01	60
58	Electron transfer flavoprotein alpha-subunit	*etfA*	0241	Q9JWU3	U(c)*	Energy metabolism (respiration)	32 526	4.82	29 907	4.87	43
59	Elongation factor Ts	*tsf*	0327	Q9JRH4	C	Synthesis and modification of macromolecules (protein translation and modification)	30 329	5.30	32 298	5.29	65
60	Putative inosine-5'-monophosphate dehydrogenase	*guaB*	1372	Q9JUD0	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	52 367	6.72	53 961	6.87	52
61	CTP synthase	*pyrG*	1742	Q9JTK1	U(c)*	Central intermediary metabolism	59 916	5.57	70 347	5.79	33
62	Putative dihydrolipoamide dehydrogenase E3 component	*lpdA3*	1151	Q9JUT1	C*	Energy metabolism (pyruvate dehydrogenase)	50 135	5.90	52 782	6.03	40
63	Bifunctional purine biosynthesis protein purH	*purH*	1182	Q9JUQ8	U*	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	57 435	5.85	55 384	6.40	41
64	Porin B	*porB*	0398	P57042	IN/OM	Cell envelope (membranes, lipoprotein and porins)	35 543	7.11	32 065	6.84	71
65	Succinyl-CoA synthetase alpha subunit	*sucD*	1154	Q9JUS9	U(c)*	Energy metabolism (tricarboxylic acid cycle)	30 579	5.79	32 168	6.33	53

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[a]	Cell[a]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
66	Putative cysteine synthase	*cysK*	0974	Q9JQL6	U(c)*	Amino acids biosynthesis (serine family)	32 820	6.06	32 581	6.47	65
67	ATP synthase beta chain	*atpD*	0519	Q9JW70	U(c)*	Energy metabolism (ATP-proton motive force)	50 394	4.92	48 203	4.94	58
68	Outer membrane protein Omp85	*omp85*	0085	Q9JX31	OM*	Cell envelope (membranes, lipoprotein and porins)	88 404	8.65	80 374	nd	17
69	Putative dihydro-lipoamide dehydrogenase	*lpdA2*	1142	Q9JUT5	C*	Energy metabolism (pyruvate dehydrogenase)	50 747	5.95	51 271	6.66	37
70	Putative 2-oxoglutarate dehydrogenase E1 component	*sucA*	1149	Q9JRJ8	U(c)*	Energy metabolism (tri-carboxylic acid cycle)	105 082	6.24	nd	6.68	31
71	Putative isocitrate dehydrogenase	*icd*	1116	Q9JUV7	U*	Energy metabolism (tri-carboxylic acid cycle)	79 995	5.57	77 781	5.58	45
72	Aspartyl-tRNA synthetase	*aspS*	2019	Q9JT23	C	Central intermediary metabolism	67 976	5.38	72 243	5.38	47
73	Biosynthetic arginine decarboxylase	*speA*	2017	Q9JT25	PS*	Central intermediary metabolism	70 901	5.46	72 779	5.60	16
76	Threonyl-tRNA synthetase	*thrS*	0929	Q9JVA3	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	72 689	5.75	75 852	6.00	26
77	Putative amidophospho-ribosyltransferase	*purF*	0892	Q9JVC9	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	56 010	5.96	55 384	6.09	29
78	Putative acetyl-CoA carboxylase biotin carboxylase component	*accC*	0596	Q9JW07	U(c)*	Fatty acid biosynthesis	49 657	5.83	56 758	5.26	48
79	Fumarate hydratase class II	*fumC*	1670	Q9JTR0	C*	Energy metabolism (tri-carboxylic acid cycle)	49 391	5.83	46 993	6.21	44
80	Putative zinc-binding alcohol dehydrogenase		0808	Q9JVJ8	INN*	Degradation (carbon compounds)	37 888	5.31	44 361	5.32	53
81	Glutamine synthetase	*glnA*	2128	Q9JSU6	C*	Amino acids biosynthesis (glutamate family)	52 076	5.20	61 496	5.25	46
82	O-succinylhomoserine sulfhydrolase	*metZ*	1808	Q9JTE7	C*	Amino acids biosynthesis (aspartate family)	41 986	6.27	39 752	6.74	51
83	Putative ABC-transporter ATP-binding protein		1409	Q9JUB3	INN*	Transport/binding proteins (other transporter)	60 723	5.04	66 901	4.99	47
84	Septum site-determining protein	*minD*	0100	Q9JQY6	U(c)*	Cell division	29 559	5.70	27 088	5.97	44
85	Oligopeptidase A	*prlC*	0054	Q9JX57	C*	Degradation of macromolecules (proteins, peptides and glycopeptides)	76 013	5.20	70 660	5.00	38
86	Dihydrolipoamide dehydrogenase	*lpdA*	1556	Q9JU06	C*	Energy metabolism (pyruvate dehydrogenase)	62 105	5.07	72 136	4.94	41
87	Putative GTP-binding protein	*typA*	1370	Q9JUD2	C*	Broad regulatory functions	67 257	5.04	72 779	4.99	61

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[a]	Cell[a]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)	
88	Dihydrolipoamide *S*-acetyltransferase component of pyruvate dehydrogenase complex	*aceF*	1555	Q9JU07	INN*	Energy metabolism (pyruvate dehydrogenase)	55 174	5.23	75 072	5.07	28	
89	Putative aspartate aminotransferase	*aspC*	0719	Q9JVS3	C*	Amino acids biosynthesis (aspartate family)	44 016	5.69	39 240	6.09	49	
90	Carbamoyl-phosphate synthase small chain	*carA*	0608	Q9JV26	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (pyrimidine ribonucleotide biosynthesis)	40 604	5.45	42 311	5.36	34	
91a	Trigger factor	*tig*	1526	Q9JU32	U(c)*	Transport/binding proteins (other transporter)	48 326	4.78	52 591	4.71	46	
91b	Putative phosphate acyltransferase		0841	Q9JVH2	U(c)*	Degradation (carbon compounds)	52 187	4.79	52 591	4.71		
92	Thioredoxin reductase	*trxB*	1538	Q9JU23	C*	Purines, pyrimidines, nucleosides and nucleotides (2'-deoxyribonucleotide metabolism)	33 702	5.16	29 051	5.03	66	
93	Putative malonyl CoA-acyl carrier protein transacylase	*fabD*	0536	Q9JW58	U(c)*	Fatty acid biosynthesis	31 899	5.11	28 365	5.02	53	
94	Hypothetical protein NMA0604		0604	Q9JW00	U*	Conserved hypothetical protein	21 221	6.52	17 539	6.88	77	
95	Putative regulator of PilE expression	*regF*	0498	Q9JW85	U(c)*	Broad regulatory functions	23 106	6.53	21 621	6.85	64	←
97	Putative ferredoxin-NADP reductase	*fpr*	1442	Q9JRE3	U(c)*	Energy metabolism (respiration)	29 313	5.73	24 682	6.00	60	←
98	Putative UDP-*N*-acetyl-D-glucosamine 2-epimerase	*sacA*	0199	O68214	U*	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	41 703	5.91	38 237	6.26	41	
99	Aminomethyltransferase	*gtvT*	0758	Q9JVP2	U(c)*	Degradation (amino acids and amines)	39 910	5.95	37 844	6.19	38	
100	Aldehyde dehydrogenase A	*aldA*	0480	Q9JW97	C*	Energy metabolism (fermentation)	52 255	5.26	53 143	5.28	40	
101	Argininosuccinate synthase	*argG*	0303	Q9JWM1	C	Amino acids biosynthesis (glutamate family)	46 757	5.24	51 086	5.28	64	
102	Aspartyl-glutamyl-tRNA (Asn/Gln) amidotransferase subunit B	*gatB*	1570	Q9JTZ3	U(c)*	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	51 876	5.22	52 401	5.25	49	
103	Phosphoglycerate kinase	*pgk*	0257	Q9JWS8	C	Energy metabolism (glycolysis)	40 661	5.17	40 165	5.02	30	
104	Putative polyamine permease substrate-binding protein		2023	Q9JT20	PS*	Transport/binding proteins (amino acids and amines)	41 357	5.54	38 736	5.05	41	

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
105	RecA protein	*recA*	1658	P56987	C	Synthesis and modification of macromolecules (DNA replication, restriction/modification, recombination and repair)	37 612	5.11	37 844	5.05	69
106	DNA-directed RNA polymerase alpha subunit	*rpoA*	0103	Q9JR06	U(c)*	Synthesis and modification of macromolecules (RNA synthesis, RNA modification and DNA transcription)	36 076	4.94	39 649	4.93	45
107	Succinyl-CoA synthetase beta chain	*sucC*	1153	Q9JUT0	U(c)*	Energy metabolism (tricarboxylic acid cycle)	41 323	5.07	38 436	4.93	26
108	Dihydrolipoamide putative succinyltransferase E2 component	*sucB*	1150	Q9JUT2)	INN*	Energy metabolism (tricarboxylic acid cycle)	41 516	5.14	39 752	4.96	33
109	Succinyl-CoA synthetase beta chain	*sucC*	1153	Q9JUT0	U(c)*	Energy metabolism (tricarboxylic acid cycle)	41 323	5.07	38 139	4.96	33
110	Succinyl-CoA synthetase beta chain	*sucC*	1153	Q9JUT0	U(c)*	Energy metabolism (tricarboxylic acid cycle)	41 323	5.07	37 260	4.95	30
111a	Transaldolase	*tal*	2136	Q9JSU1	C	Energy metabolism (pentose phosphate patway)	37 317	5.09	34 476	5.10	39
111b	Aspartate-semialdehyde dehydrogenase	*asd*	0351	P57008	U*	Amino acids biosynthesis (aspartate family)	40 043	5.30	35 108	5.28	39
112	Adenylosuccinate synthetase	*purA*	1024	Q9JV25	C	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	45 946	5.77	48 731	6.00	37
113	Putative cysteine synthase	*cysK*	0974	Q9JQL6	U(c)*	Amino acids biosynthesis (serine family)	32 820	6.06	32 399	6.06	45
114	Putative pilus retraction protein	*pilT2*	0979	Q9JV63	C*	Cell envelope (surface structures)	41 419	6.15	39 752	6.54	53
115	L-lactate dehydrogenase	*lldA*	1592	Q9JTX1	U*	Energy metabolism (respiration)	43 486	6.98	41 006	6.87	40
116	Putative periplasmic protein	0165		Q9JWY8	U*	Cell envelope (periplasmic proteins)	45 582	7.78	44 593	6.87	64
117	Glutamyl-tRNA synthetase	*gltX*	0250	Q9JWT4	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	52 470	5.46	55 535	5.54	34
118	Glucose 6-phosphate 1-dehydrogenase	*zwf*	1609	Q9JTW0	U(c)*	Energy metabolism (pentose phosphate patway)	54 108	5.36	53 361	5.53	44
119	Glutamyl-tRNA(Gln) amidotransferase subunit A	*gatA*	1568	Q9JTZ5	U*	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	51 338	5.55	51 458	5.59	31

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
120	Seryl-tRNA synthetase	*serS*	1943	Q9JT77	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	47 962	5.60	50 532	5.89	69
121	Chaperone protein dnaK	*dnaK*	0736	Q9JVQ9	U(c)*	Transport/binding proteins (other transporters)	68 906	4.79	74 080	4.74	29
122	ABC transporter ATP-binding protein		2101	Q9JSW5	INN*	Transport/binding proteins (other transporters)	62 086	5.23	68 907	5.38	54
123	Putative aminopeptidase		1640	Q9JTT6	U(c)*	Degradation of macromolecules (proteins, peptides and glycopeptides)	71 764	5.70	68 098	5.31	26
124	Pyruvate kinase	*pykA*	0177	Q9JWX8	U(c)*	Energy metabolism (glycolysis)	52 465	5.33	67 597	5.35	49
125	Putative succinate dehydrogenase flavoprotein subunit	*sdhA*	1145	Q9JUT3	PS*	Energy metabolism (tricarboxylic acid cycle)	64 460	5.89	69 521	6.01	19
126	Acetolactate synthase isozyme III large subunit	*ilv1*	1766	Q9JTI1	U*	Amino acid biosynthesis (branched chain family)	62 784	5.88	65 726	5.96	42
127	Pyruvate dehydrogenase E1 component	*aceE*	1554	Q9JU08	U*	Energy metabolism (pyruvate dehydrogenase)	99 563	5.56	84 547	5.84	48
129	Transketolase	*tkt*	1669	Q9JTR1	U(c)*	Energy metabolism (pentose phosphate patway)	71 743	5.44	74 409	5.46	49
132	Putative lipoprotein		0281	Q9JWN6	U(c)*	Cell envelope (membrane, lipoproteins and porins)	42 280	4.80	44 131	4.66	54
134	Probable D-lactase dehydrogenase	*dld*	1205	Q9JUP8	U(c)*	Energy metabolism (respiration)	63 428	6.32	69 727	6.77	29
135	60 kDa chaperonin	*groEL*	0473	P57006	C	Transport/binding proteins (other transporters)	57 456	4.90	65 532	4.83	66
137a	Methionyl-tRNA synthetase	*metG*	0275	Q9JWP0	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	76 984	5.36	74 409	5.35	32
137b	Transketolase	*tkt*	1669	Q9JTR1	U(c)*	Energy metabolism (pentose phosphate patway)	71 743	5.44	74 409	5.35	24
140	Glucosamine-fructose-6-phosphate aminotransferase (isomerizing)	*glmS*	0278	Q9JWN9	C	Central intermediary metabolism (amino sugars)	66 440	6.02	71 817	6.37	33
141	Chromosomal replication initiator protein dnaA	*dnaA*	0552	Q9JW45	U(c)*	Synthesis and modification of macromolecules (DNA replication, restriction/modification, recombination and repair)	57 986	5.80	68 400	6.08	31
142	Dihydrolipoamide dehydrogenase	*lpdA*	1556	Q9JU06	C*	Energy metabolism (pyruvate dehydrogenase)	62 105	5.07	75 852	4.94	36

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)	
144	Enolase	*eno*	1495	Q9JU46	C	Energy metabolism (glycolysis)	46 105	4.78	44 541	4.76	46	
145	Glutaminyl-tRNA synthetase	*glnS*	1748	P57000	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	64 769	5.83	70 244	6.16	43	
146	Nutilization substance protein A	*nusA*	1896	Q9JTB6	U(c)*	Synthesis and modification of macromolecules (RNA synthesis, RNA modification and DNA transcription)	56 418	4.55	67 997	4.57	33	
147	Cell division protein ftsZ	*ftsZ*	2057	Q51130	C	Cell division	41 487	4.94	40 479	4.90	72	
148	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	*metE*	1140	Q9JUT6	U(c)*	Amino acids biosynthesis (aspartate family)	85 122	5.31	79 877	5.35	32	
149	Putative phosphoenolpyruvate synthase	*ppsA*	0826	Q9JVI5	C*	Central intermediary metabolism (gluconeogenesis)	87 208	5.01	82 335	4.92	34	
151	Type IV pilus assembly protein	*pilF*	2159	Q9JR75	C*	Cell envelope (surface structures)	61 865	5.42	66 213	5.58	46	
152	30S ribosomal protein S1	*rpsA*	1515	Q9JU38	C*	Synthesis and modification of macromolecules (ribosomal protein synthesis and modification)	61 234	4.98	68 299	4.93	63	
153	Lysyl-tRNA synthetase	*lysS*	1638	Q9JTT7	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	57 264	5.34	57 804	5.35	51	
154	GMP synthase (glutamine-hydrolyzing)	*guaA*	0534	Q9JW60	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	57 730	5.53	54 313	5.53	35	
155	Probable sulphate adenylate transferase subunit 1	*cysN*	1364	Q9JUD7	C*	Central intermediary metabolism (sulphur metabolism)	46 838	5.15	46 148	5.04	45	
156	Putative two-component system regulator		0798	Q9JRJ9	C*	Signal transduction	24 779	5.44	22 468	5.48	49	↙
157	Hypothetical protein NMA 1773		1773	Q9JTH7	U*	Conserved hypothetical protein	30 407	5.33	25 605	5.30	66	
158	Putative oxidoreductase		0666	Q9JVV3	U*	Miscellaneous	20 714	5.73	17 994	6.00	56	↙
159	Putative peptidyl-prolyl isomerase		1756	Q9JTJ0	OM*	Synthesis and modification of macromolecules (protein translation and modification)	28 909	5.72	27 227	5.27	36	
160	2,3,4,5-tetrahydropyridine-2-carboxylate *N*-succinyltransferase	*dapD*	2153	Q9JSS7	C*	Amino acids biosynthesis (aspartate family)	29 425	5.42	26 721	5.46	37	

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
161a	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	C	Energy metabolism (fermentation)	36 346	5.65	35 934	5.38	45
161b	Glyceraldehyde-3-phosphate dehydrogenase C	0246		Q9JWT8	C*	Energy metabolism (glycolysis)	35 772	5.52	35 934	5.38	
162	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	C*	Energy metabolism (fermentation)	36 346	5.65	36 027	5.85	65
163	Glutamate dehydrogenase	*gdhA*	1964	Q9JT56	U*	Amino acids biosynthesis (glutamate family)	48 462	5.80	44 515	5.83	33
164	Putative branched-chain amino acid aminotransferase	*ilvE*	2151	Q9JSS9	U(c)*	Amino acid biosynthesis (branched chain family)	36 102	5.67	34 032	5.88	24
165	Ketol-acid reducto-isomerase	*ilcV*	1763	Q9JTI3	U(c)*	Amino acid biosynthesis (branched chain family)	36 408	5.65	35 472	5.95	49
166	Putative fructose-1,6-bisphosphate aldolase	*fba*	0587	Q9JW15	U(c)*	Energy metabolism (glycolysis)	38 415	5.66	36 027	6.02	62
167	Putative polyamine permease substrate-binding protein		1786	Q9JTG5	PS*	Transport/binding protein (amino acids and amines)	41 949	5.96	38 337	5.58	45
168	Riboflavin biosynthesis protein ribA	*ribA*	1429	Q9JU97	U(c)* U(c)*	Biosynthesis of cofactor, prosthetic groups and carriers (riboflavin)	39 398	5.34	37 357	5.36	
169a	Glyceraldehyde-3-phosphate dehydrogenase C		0246	Q9JWT8	C	Energy metabolism (glycolysis)	35 772	5.52	35 934	5.59	63
	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	C*	Energy metabolism (fermentation)	36 346	5.65	35 934	5.59	46
169b	Phenylalanyl-tRNA synthetase alpha chain	*pheS*	0933	Q9JR76	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	37 305	5.46	35 934	5.59	
170	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	C*	Energy metabolism (fermentation)	36 346	5.65	36 591	5.40	32
172	Putative capsule biosynthesis protein	*sacB*	0200	Q9JWW8	U*	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	64 133	9.11	44 554	nd	27
174	Na(+)-translocating NADH-quinone reductase subunit A	*nqrA*	0752	Q9JVP8	U(c)*	Transport/binding protein (cations)	48 673	6.20	56 552	6.63	44
175a	Putative membrane transport solute-binding protein	*fstB*	0452	Q9JWB9	PS*	Transport/binding protein (cations)	34 149	5.67	32 496	4.94	30
176b	Hypothetical protein NMA0696	*rtw8*	0696	Q9JR89	U(c)*		36 476	5.32	32 496	4.94	20
178	Glycyl-tRNA synthetase alpha chain	*glyQ*	0521	Q9JRC6	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	34 209	4.98	32 231	4.98	27

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
178	Putative pilus retraction protein	*pilT*	0218	Q9JWV9	C*	Cell envelope (surface structures)	37 968	6.42	36 120	6.85	52
180	*S*-adenosylmethionine synthetase	*metK*	0663	Q9JVV6	C	Central intermediary metabolism	42 091	5.10	44 182	5.05	33
181	Putative aminotransferase		1113	Q9JUW0	U*	Miscellaneous	44 055	5.74	41 006	6.65	24
182	Ribose-phosphate pyrophosphokinase	*prsI/ prsA*	1093	Q9JQV4	C	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	35 597	5.41	32 477	5.36	46
184	L-lactate dehydrogenase	*lldA*	1592	Q9JTX1	U*	Energy metabolism (respiration)	43 486	6.98	41 167	6.82	45
	Acetylornithine aminotransferase	*argD*	1584	Q9JTX9	C	Amino acids biosynthesis (glutamate family)	42 546	6.50	41 167	6.82	15
185	Tryptophan synthase beta chain	*trpB*	0904	Q9JVC0	U*	Amino acids biosynthesis (aromatic amino acid family)	43 193	6.19	40 900	6.71	28
186	3-isopropylmalate dehydrogenase	*leuB*	1456	Q9JU79	C	Amino acid biosynthesis (branched chain family)	39 001	4.91	38 536	4.83	35
187	Delta-aminolevulinic acid dehydratase	*hemB*	1011	Q9JV37	U(c)*	Biosynthesis of cofactor, prosthetic group and carriers (heme and porphyrin)	37 277	5.23	40 062	5.27	32
188	3-oxoacyl-[acyl-carrier-protein] synthase II	*fabF*	0044	Q9JX65	U(c)*	Fatty acid biosynthesis	43 129	5.45	44 284	5.59	40
189	30S ribosomal protein S2	*rpsB*	0328	Q9JRG7	U(c)*	Synthesis and modification of macromolecules (ribosomal protein synthesis and modification)	26 898	9.04	26 858	nd	44
190	Putative type I restriction-modification system protein		1038	Q9JV16	U(c)*	Synthesis and modification of macromolecules (DNA replication, restriction/ modification, recombination and repair)	57 320	5.02	55 745	4.93	48
191	Hypothetical protein NMA0989		0989	Q9JV54	U*	Conserved hypothetical protein	46 301	5.26	54 709	5.02	39
193	Putative formate-tetrahydrofolate ligase	*fhs*	0617	Q9JVY8	U(c)*	Central intermediary metabolism	59 122	5.85	61 400	6.30	29
194	Bifunctional purine biosynthesis protein purH	*purH*	1182	Q9JU08	U*	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	57 435	5.85	54 313	6.28	31
195	Argininosuccinate lyase	*argH*	0847	Q9JVG7	C	Amino acids biosynthesis (glutamate family)	51 286	5.28	44 990	5.30	33
	Putative homoserine dehydrogenase	*hom*	1395	Q9JR84	U(c)*	Amino acids biosynthesis (aspartate family)	46 545	5.31	44 990	5.30	39
196	Putative periplasmic serine protease		0710	Q9JVT1	U(ps)*	Degradation of macromolecules (proteins, peptides and glycoproteins)	52 563	5.31	57 379	4.82	46

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
197	Hypothetical protein NMA0866		0866	Q9JVF0	U*	Unknown	47 027	6.02	51 645	6.47	28
198	Putative fructose-1,6-bisphosphatase	*fbp*	1259	Q9JUL6	U(inn)*	Central intermediary metabolism (gluconeogenesis)	35 550	5.49	32 821	5.60	47
199	Putative dihydrolipoamide dehydrogenase	*lpdA2*	1142	Q9JUT5	C*	Energy metabolism (pyruvate dehydrogenase)	50 747	5.95	51 271	6.54	48
200	Putative replicative DNA helicase	*dnaB*	1105	Q9JUW8	U(c)*	Synthesis and modification of macromolecules (DNA replication, restriction/modification, recombination and repair)	52 080	5.04	53 167	4.92	38
201	Phosphoglucomutase	*pgm*	1001	P57002	U*	Degradation (carbon compounds)	49 499	5.26	52 591	5.12	27
202	Phosphoribosylaminoimidazole-succinocarboxamide synthase	*purC*	0968	Q9JV73	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	32 251	5.26	29 754	5.27	60
203	Lactoferrin-binding protein	*lbpB*	1740	Q9JTK3	U*	Transport/binding protein (cations)	81 559	4.63	81 208	4.77	25
204	Adenylate kinase	*adk*	1032	P49980	C	Purines, pyrimidines, nucleosides and nucleotides (purine ribonucleotide biosynthesis)	23 216	5.02	25 128	4.95	50
205	Histidine-binding periplasmic protein	*hisJ*	1811	Q9JTE4	PS*	Transport/binding protein (amino acids and amines)	29 029	5.25	25 736	4.80	74
206	Outer membrane protein class 4	*rmpM*	2105	P38367	IN/OM	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	26 140	6.53	30 945	6.33	28
207	Outer membrane protein class 4	*rmpM*	2105	P38367	IN/OM	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	26 140	6.53	31 157	6.22	33
208	Aromatic amino acid aminotransferase	*tyrB*	1937	Q9JT83	C*	Amino acids biosynthesis (aromatic amino acid family)	44 640	5.30	39 038	5.30	41
209	ATP phosphoribosyltransferase regulatory subunit	*hisZ*	1023	Q9JV26	C	Conserved hypotetical protein	41 637	5.38	39 649	5.53	46
210	Glutamate-1-semialdheyde 2,1-aminomutase	*hemL*	0592	Q9JW10	C	Biosynthesis of cofactor, prosthetic group and carriers heme and porphyrin)	45 268	5.56	39 546	5.79	63
211	Outer membrane class protein 4	*rmpM*	2105	P38367	IN/OM	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	26 140	6.53	30 998	6.02	28

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
212	Ribose-phosphate pyrophosphokinase	*prs/ prsA*	1093	Q9JQV4	C	Purines, pyrimidines,nucleosides and nucleotides (purine ribonucleotide biosynthesis)	35 597	5.41	34 835	5.77	32
213	Probable malate:quinone oxidoreductase	*mqo*	0333	Q9JWK3	U*	Energy metabolism (tricarboxylic acid cycle)	53 993	5.67	52 782	5.93	47
214	Probable malate:quinone oxidoreductase	*mqo*	0333	Q9JWK3	U*	Energy metabolism (tricarboxylic acid cycle)	53 993	5.67	52 782	5.93	30
215	DNA helicase II	*uvrD*	0027	Q9JR27	U(c)*	Synthesis and modification of macromolecules (DANN replication, restriction/modification, recombination and repair)	82 297	6.17	77 323	6.22	19
216	Putative UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	*mpl*	1356	Q9JUE5	U(c)*	Cell envelope (murein sacculus and peptidoglycan)	49 385	5.94	44 173	6.28	39
217	Putative tyrosyl-tRNA synthetase	*tyrS*	0620	Q9JVY6	C*	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	47 329	5.80	44 157	6.33	30
218	Putative phosphoglucomutase/phosphomannomutase		1949	Q9JT71	U(c)*	Central intermediary metabolism	47 851	5.54	46 993	5.43	35
219	Hypotetical protein NMA1808		1808	Q9JTA4	U(c)*	Conserved hypothetical protein	46 471	4.95	34 476	4.85	20
221	Pilus assembly protein	*pilO*	0652	Q9JR13	U(inn)*	Cell envelope (surface structure)	23 315	5.04	19 767	4.94	51
222	Haem utilization protein	*hemO*	1927	Q9RGD9	U(c)*	Transport/binding protein (cations)	25 792	6.00	21 714	6.02	47
223	Putative NAD(P)H-flavin oxidoreductase		1015	Q9JV33	U*	Miscellaneous	24 795	5.79	20 717	5.95	44
224	Putative 3-oxoacyl-(acyl-carrier protein) reductase	*fabG*	0533	Q9JW61	U(c)*	Fatty acid biosynthesis	25 987	5.58	20 629	5.84	71
225	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*gmpA*	1801	Q9JTF2	U(c)*	Energy metabolism (glycolysis)	26 000	5.59	22 953	5.84	74
226	Single-strand binding protein	*ssb*	1672	Q9JRF8	U*	Synthesis and modification of macromolecules (DNA replication, restriction/ modification, recombination and repair)	19 452	5.76	14 975	6.14	82
227	Transcription antitermination protein NusG	*nusG*	0147	Q9JRD9	U(c)*	Synthesis and modification of macromolecules (RNA synthesis, RNA modification and DNA transcription)	20 550	6.03	16 592	6.34	79

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
228	Ribosome recycling factor	*frr*	0080	Q9JR52	C	Synthesis and modification of macromolecules (protein translation and modification)	20 730	6.22	16 034	6.59	42
229	Chorismate synthase	*aroC*	1939	Q9JT81	U(c)*	Amino acids biosynthesis (aromatic amino acid family)	39 413	6.06	37 746	6.57	32
230	Glyceraldehyde-3-phosphate dehydrogenase	*gapA*	0062	Q9JX51	C	Energy metabolism (glycolysis)	37 022	6.08	38 636	6.69	35
231	Aconitate hydratase	*acnB*	1761	Q9JTI5	U*	Energy metabolism (tricarboxylic acid cycle)	92 725	5.42	86 321	5.46	38
232	Putative isoleucyl-tRNA synthetase	*ileS*	0622	Q9JVY4	C*	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	104 185	5.60	nd	5.77	16
233	Glycyl-tRNA synthetase beta chain	*glyS*	0523	Q9JW67	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	74 650	5.30	75 405	5.31	20
234	Putative polyribonucleotide nucleotidyltransferase	*pnp*	0969	Q9JV72	C*	Degradation of macromolecules (RNA)	76 258	5.35	76 640	5.33	21
235	ClpB protein	*clpB*	1683	Q9JTP9	U(c)*	Degradation of macromolecules (proteins, peptides and glycopeptides)	95 093	5.45	77 896	5.32	20
237	Leucyl-tRNA synthetase	*leuS*	0559	Q9JW39	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	101 505	5.20	87 093	5.15	20
238	Putative malate oxidoreductase	*maeA*	0870	Q9JVE6	U(c)*	Central intermediary metabolism (gluconeogenesis)	45 991	5.08	46 148	4.97	56
239	Histidyl-tRNA synthetase	*hisS*	1065	Q9JUZ9	C	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	48 474	5.47	44 215	5.69	34
240	Putative GTP-binding protein		0345	Q9JWJ4	U(c)*	Miscellaneous	41 968	5.61	44 438	5.66	26
241	Glutamate dehydrogenase	*gdhA*	1964	Q9JT56	U*	Amino acids biosynthesis (glutamate family)	48 462	5.80	44 438	5.53	32
242	UDP-3-O-[3-hydroxymyristoyl] *N*-acetylglucosamine deacetylase	*lpxC*	0263	Q9JWS2	U(c)*	Synthesis and modification of macromolecules (ribosomial protein synthesis and modification)	34 000	5.14	32 315	5.17	63
243	Thiamine biosynthesis protein thiC	*thiC*	0397	Q9JWF3	U(c)*	Biosynthesis of cofactor, prosthetic groups and carriers (thiamine)	71 176	6.05	73 644	6.51	40
244	Putative succinate dehydrogenase flavoprotein subunit	*sdhA*	1145	Q9JUT3	PS*	Energy metabolism (tricarboxylic acid cycle)	64 460	5.89	69 418	6.26	22

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
245	Transcription termination factor	*rho*	0825	Q9JVI6	U(c)*	Synthesis and modification of macromolecules (RNA synthesis, RNA modification and DNA transcription)	47 322	6.22	49 623	6.72	28
246	33kDa chaperonin	*hslO*	0441	Q9JWC8	C	Conserved hypotetical protein	33 264	4.69	32 213	4.70	49
247	Porin B	*porB*	0398	P57042	IN/OM	Cell envelope (membranes, lipoprotein and porins)	35 543	7.11	32 651	6.77	59
249	DNA mismatch protein mutL	*mutL*	1655	Q9JTS2	U(c)*	IS, phage-related function and prophage	71 842	5.90	77 437	6.42	25
250	Hypothetical protein NMA0194		0194	P57072	U(c)*	Synthesis and modification of macromolecules (RNA synthesis, RNA modification and DNA transcription)	83 161	5.99	81 544	6.40	28
251	Prolyl-tRNA synthetase		1553	Q9JU09	C*	Synthesis and modification of macromolecules (aminoacyl tRNA synthetases and their modification)	62 981	5.07	69 214	4.99	45
	DNA recombination protein rmuC homolog	*rmuC*	0386	Q9JWG3	INN*	Cell envelope (periplasmic proteins)	66 747	5.18	69 214	4.99	25
252	Hypothetical protein NMA1544		1544	Q9JU17	U*		29 410	6.71	25 257	6.86	48
253	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase	*folD*	0354	Q9JWI9	U(c)*	Biosynthesis of cofactor, prosthetic groups and carriers (folic acid)	30 139	5.42	31 856	5.64	48
254	Pilus assembly protein	*pilP*	0651	Q9JQN6	U*	Cell envelope (surface structures)	20 067	4.94	12 624	4.48	81
255	Phosphoglycolate phosphatase (PGP)	*gph*	1688	Q9JTP5	U(c)*	Miscellaneous	25 527	4.68	19 349	4.56	38
257	Putative acetyl-CoA carboxylase biotin carboxyl carrier protein	*accB*	0597	Q9JQT3	U(c)*	Fatty acid biosynthesis	15 679	4.67	17 242	4.60	58
258	Peptidyl-prolyl *cis-trans* isomerase B	*ppiB*	1002	Q9JQS5	C*	Synthesis and modification of macromolecules (protein translation and modification)	18 852	5.04	14 659	4.95	89
259	ATP synthase delta chain	*atpH*	0518	Q9JW73	C*	Energy metabolism (ATP-proton motive force)	19 470	5.02	13 344	4.93	79
260	Conserved hypothetical protein NMA2008		2008	Q9JR11	U(c)*	Conserved hypothetical protein	19 770	5.19	13 886	5.15	47
261	Hypothetical protein NMA1203		1203	Q9JUP9	U(c)*	Conserved hypothetical protein	19 166	5.22	13 007	5.15	46
262	Hypothetical protein NMA0693		0693	Q9JRA0	U(c)*	Unknown	19 571	5.12	12 410	4.94	42
263	Hypothetical protein NMA0996		0996	Q9JV47	U(c)*	Conserved hypothetical protein	32 783	5.26	30 111	5.30	34

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
264	Hypothetical protein NMA1809		1809	Q9JTE6	U(c)*	Unknown	28 693	5.36	30 840	5.50	20
265	Agmatinase	*speB*	2016	Q9JRG2	U(c)*	Central intermediary metabolism	33 905	5.49	29 002	5.57	27
266	Triosephosphate isomerase	*tpiA*	0570	Q9JW31	U(c)*	Energy metabolism (glycolysis)	27 530	5.38	26 675	5.55	53
267	ATP-dependent Clp protease proteolytic subunit	*clpP*	1525	Q9JU33	C	Degradation of macromolecules (proteins, peptides and glycopeptides)	22 603	5.05	17 168	5.08	41
268	Hypothetical protein NMA2195	*Ycl026 C-A*	2195	Q9JQW5	U*	Conserved hypothetical protein	22 108	5.37	16 592	5.39	65
269	Peptide deformylase (PDF)	*def*	0164	Q9JQN0	U(c)*	Synthesis and modification of macromolecules (protein translation and modification)	19 115	5.60	15 763	5.88	53
270	Dihydrodipicolinate reductase	*dapB*	0066	Q9JX48	C	Amino acids biosynthesis (aspartate family)	28 274	5.78	25 692	6.03	71
271	Phosphomethyl-pyrimidine kinase	*thiD*	1815	Q9JTE1	U(c)*	Biosynthesis of cofactor, prosthetic groups and carriers (thiamine)	28 416	6.24	25 257	6.06	51
272	Inositol-1-mono-phosphatase	*suhB*	1559	Q9JU03	U(c)*	Conserved hypothetical	28 496	5.82	26 129	6.22	30
273	Orotate phosphoribosyl-transferase	*pyrE*	0582	Q9JR25	U*	Purines, pyrimidines, nucleosides and nucleotides (pyrimidine ribonucleotide biosynthesis)	23 261	5.98	19 432	6.30	70
274	Enoyl-ACP reductase	*fabI*	2152	Q9JSS8	U*	Fatty acid biosynthesis	27 632	5.99	26 001	6.47	68
275	Uridylate kinase	*pyrH*	0326	Q9JQT5	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (pyrimidine ribonucleotide biosynthesis)	25 699	6.17	24 682	6.73	53
276	TOU3	*tou3*	2036	Q9RQW0	U*	Unknown	29 564	6.38	30 525	6.74	31
277	Adhesin	*mafA*	0325	Q9JWK7	U*	Pathogenicity	34 753	7.07	30 525	6.83	62
278	Acetyl-CoA carboxylase, carboxyl transferase alpha subunit	*accA*	1349	Q9JUF0	U(c)*	Fatty acid biosynthesis	35 492	6.41	30 787	6.85	34
279	Outer membrane protein class 4	*rmpM*	2105	P38367	IN/OM	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	26 140	6.53	29 652	6.85	24
280	Putative nuclease		0348	Q9JR99	U*	Degradation of macromolecules (DNA)	29 658	8.48	28 124	6.83	54
281	Putative lipoprotein		0586	Q9JW16	U*	Cell envelope (membranes, lipoproteins and porins)	29 765	7.73	27 227	6.75	52

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
282	Methionine aminopeptidase	*map*	0337	Q9JWK1	U(c)*	Synthesis and modification of macromolecules (protein translation and modification)	28 236	6.11	27 601	6.62	43
283	Putative gntR-family transcriptional regulator		1751	Q9JRE6	U(c)*	Broad regulatory function	27 198	6.01	26 812	6.52	73
284	Dihydrodipicolinate synthase	*dapA*	1124	Q9JUU9	C	Amino acids biosynthesis (aspartate family)	30 806	5.49	29 958	5.94	50
285	Putative hydroxyacyl-glutathione hydrolase		0444	Q9JWC6	U(c)*	Central intermediary metabolism	27 821	5.81	28 854	5.96	20
286	3-deoxy-o-manno-octulosonate cytidylyltransferase	*kdsB*	0875	Q9JVE3	U(c)*	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	27 793	5.86	29 350	6.33	44
287	2-dehydro-3-deoxy-phosphooctonate aldolase	*kdsA*	1493	Q9JU48	C	Cell envelope (surface polysaccharides, lipopolysaccharides and antigens)	30 536	5.77	29 754	6.14	38
288	Superoxide dismutase	*sodC*	1617	P57005	PS	Cell envelope (periplasmic proteins)	19 550	6.12	nd	6.57	40
289	Hypothetical protein NMA0963		0963	Q9JV77	U(c)*	Conserved hypothetical protein	18 673	6.96	nd	6.47	55
290	Hypothetical protein NMA1703		1703	Q9JTN1	U(c)*	Conserved hypothetical protein	16 551	5.27	nd	5.32	37
291	Putative marR-family transcriptional regulator		0613	Q9JR77	U(c)*	Broad regulatory function	16 583	5.14	nd	5.29	71
292	Putative oxidoreductase		1120	Q9JUV3	C*	Miscellaneous	30 119	5.10	27 743	5.07	23
294	Competence lipoprotein comL	*comL*	0907	Q9JVB7	U*	Transport/binding protein (other transporters)	30 808	8.72	25 474	nd	46
295	Ornithine carbamoyl-transferase	*argF*	1762	Q9JYI4	C	Amino acids biosynthesis (glutamate family)	36 703	5.86	36 308	6.22	44
296	Putative glycerate dehydrogenase		0274	Q9JWP1	C*	Amino acids biosynthesis (serine family)	34 790	6.18	34 655	6.22	28
298	3-oxoacyl-[acyl-carrier-protein] syntase III	*fabH*	0538	Q9JW56	C	Fatty acid biosynthesis	33 833	5.97	32 439	6.27	43
300	Amino acid ABC transporter, ATP-binding protein		0900	Q9JVC3	C*	Transport/binding protein (cations)	27 041	5.93	23 650	6.30	50
301	DNA-directed RNA polymerase beta chain	*rpoB*	0142	P57009	U(c)*	Synthesis and modification of macromolecules (RNA synthesis, RNA modification and DNA transcription)	155 576	5.36	nd	5.08	16
302	Ribonucleoside-diphosphate reductase I large chain	*nrdA*	1501	Q9JU43	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (2'-deoxyribonucleotide metabolism)	85 151	5.82	79 759	6.23	20

Table 1. Continued

Spot	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Cell[c]	Functional classification	Theor. M_r (Da)	Theor. pI (pH)	Exp. M_r (Da)	Exp. pI (pH)	Sequence coverage (%)
303	NADH dehydrogenase I chain G	*nuoG*	0010	Q9JX87	U(c)*	Energy metabolism (respiration)	81 490	5.88	78 706	6.23	19
304	Carbamoyl phosphate synthase large chain	*carB*	0602	Q9JW02	U(c)*	Purines, pyrimidines, nucleosides and nucleotides (pyrimidine ribonucleotide biosynthesis)	117 419	5.10	nd	5.08	27
305	Preprotein translocase SecA subunit		1536	Q9JYK8	C*	Synthesis and modification of macromolecules (DNA replication, restriction/ modification, recombination and repair)	103 294	5.05	nd	5.03	22
306	Serine hydroxymethyltransferase	*glyA*	1254	Q9XAY7	C	Amino acids biosynthesis (serine family)	44 987	6.32	44 341	6.65	37
307	Putative amino acid permease substrate-binding protein		0997	Q9JV46	PS*	Transport/binding protein (amino acids and amines)	28 869	5.65	24 893	4.94	63
308	Glutaredoxin		1141	Q9JQ54	U*	Detoxification	26 911	4.80	27 042	4.90	40
309	Outer membrane lipoprotein GNA1946		1946	Q9JPI7	U*	Conserved hypothetical protein	31 248	5.03	26 950	4.72	43
310	Glutaredoxin		1141	Q9JQ54	U*	Detoxification	26 911	4.80	26 696	4.80	64
	Outer membrane lipoprotein GNA1946		1946	Q9JPG6	U*	Conserved hypothetical protein	31 314	5.19	26 696	4.80	61
311	Putative zinc-binding alcohol dehydrogenase		0808	Q9JVJ8	INN*	Degradation (carbon compounds)	37 888	5.31	44 336	5.28	31
312	UDP-*N*-acetylmuramate-L-alanine ligase	*murC*	2061	Q9JSZ8	C	Cell envelope (murein sacculus and peptidoglycan)	50 338	5.69	55 133	6.00	27

a) Gene number (NMAxxxx)
b) Swiss-Prot/TrEMBL accession number
c) Subcellular localization; U: unknown, C: cytoplasm, IN/OM: inter-membrane, OM: outer membrane, INN: inner membrane; PS: periplasm. The asterisk indicates that the predicted cellular localization was obtained by using PSORT-B program (cut-off value 7.5), otherwise the subcellular localization was obtained directly from the Swiss-Prot entry. When all the possibilities were equally probable the U notation (= unknown) was adopted; however, according to the PSORT-B notation, next to U (parenthesis, and small case) a localization which was more probable than others is also reported
nd: not determined

Forty-one proteins were found to be present as multiple electrophoretic species, as reported in Table 3. Heterogeneity was mainly due to variability in pI values (horizontal spot patterns), but also variability in apparent M_r or a combination of both types of value, were observed (vertical and diagonal spot patterns, respectively) . It is worth noting that the majority of them (32 gene products) have either proven or putative catalytic activities (particularly dehydrogenases and synthases/synthetases), because it is known that isozymes provide a better efficiency in biocatalysis. Two (diagonal pattern) protein species were found for the ClpB protein (spots 30 and 235), never previously isolated from *Neisseria*. ClpB is part of the ClpB/DnaK/DnaJ/GroE chaperone machinery and has a fundamental role in protein disaggregation. Particularly, ClpB transiently interacts with protein aggregates and then forms a species-specific complex with DnaK (spot 121 in Table 1) to efficiently perform resolubilization of the aggregates [30]. Multiple species (horizontal pattern) were found for several outer membrane proteins like porin A, porin B, RmpM, Omp85, all of which are predicted to be lipoproteins. An example of a vertical spot pattern is that observed in the case of dihydrolipoamide dehydrogenase discussed above.

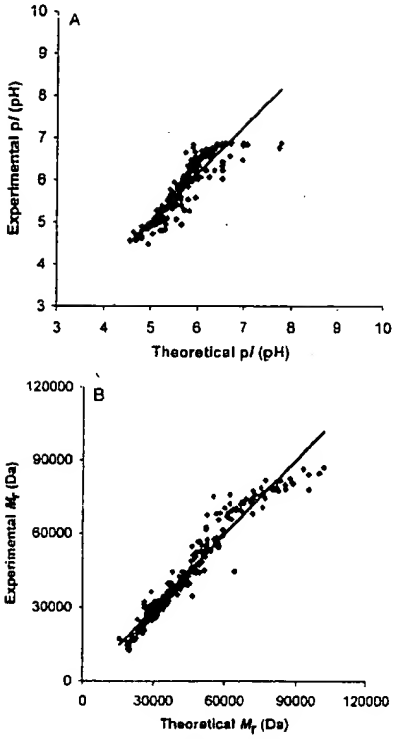


Figure 4. Comparison of experimentally determined and theoretical p/ (A) and M_r (B) values of identified protein spots from *N. meningitidis* 2-DE gels. Overall a fairly good correlation is observed for the majority of the data which fall in the M_r window between 20 and 80 kDa and the p/ window between 4.5 and 6.5.

3.3 Functional classification of identified proteins

In Table 1 the identified proteins are annotated also according to their predicted functional classification [20]. Many of the proteins in Table 1 were defined in the genome sequence annotation as hypothetical or putative and the present work provides the first biochemical confirmation of their actual expression. The more represented functional classes are briefly discussed below.

Table 2. The twenty *N. meningitidis* proteins with the highest volume in the 2-DE gel

Spots	Acc.	Protein name (SW/Tr)	Relative vol.%
57	Q9JRI5	Elongation factor Tu	4335
161a, 169a, 162, 170	Q9JVR8	Putative alcohol dehydrogenase	2724
66, 113	Q8J0L6	Putative cysteine synthase	2271
64, 247	O53989	PorB protein	2000
135	P57006	60 KDa chaperonin	1867
52, 163, 241	Q9JT56	Glutamate dehydrogenase	1805
50, 206, 207, 211, 279	P38367	Outer membrane protein class 4	1682
58	Q9JX07	Elongation factor G	1538
80, 311	Q9JVJ8	Putative zinc-binding alcohol dehydrogenase	1392
152	Q9JU38	30S ribosomal protein S1	1288
127	Q9JU08	Pyruvate dehydrogenase E1 component	1205
132	Q9JWN6	Putative lipoprotein	1082
121	Q9JVQ9	Chaperone protein dnaK	1042
54, 55a	Q9JTM0	Acetate kinase 1	0.960
149	Q9JVI5	Putative phosphoenolpyruvate synthase	0.898
165	Q9JTI3	Ketol-acid reductoisomerase	0.895
81, 15	Q9JSU6	Glutamine synthetase	0.870
53	Q9JW72	ATP synthase alpha chain	0.811
148	Q9JUT6	5-methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase	0.734
65	Q9JUS9	Succinyl-CoA synthetase alpha subunit	0.525

Protein spots were quantified by integrating optical density over Gaussian area using the Melanie II software

3.3.1 Small molecule metabolism

Energy metabolism is the functional class which is most represented in the map, accounting for 46 proteins (multiple protein species excluded), and covering around 17% of the identified protein species. Within this class we detected the expression of enzymes of glycolysis, the pyruvate dehydrogenase system, the tricarboxylic acid cycle (TCA), the pentose phosphate pathway, respiration, fermentation and ATP-proton motive force. The highest number of enzymes belong to the pyruvate dehydrogenase system and TCA, which play a fundamental role in the catabolism of glucose in *N. meningitidis* [31]. Comparison of biochemical proteomic data with *in silico*

Table 3. *N. meningitidis* proteins expressed as multiple protein species

Protein	Gene	Acc.	Spot or spot series	Type of isoform		
				Horizontal	Vertical	Diagonal
Transferrin-binding protein B	*tbpB*	O68937	47a, 47b	X		
Putative alcohol dehydrogenase*	*adhA*	Q9JVR8	161, 162, 169, 170	X		
Porin A*	*porA*	Q9JPT6	39, 40,	X		
Acetate kinase 1	*ackA1*	Q9JTM0	54, 55	X		
Glyceraldehyde-3-phosphate dehydrogenase C		Q9JWT8	161, 169	X		
Glutamate dehydrogenase*	*gdhA*	Q9JT56	52, 163, 241	X		
Ribose-phosphate pyrophosphokinase*	*prs*	Q9JQV4	182, 212			X
Succinyl-CoA synthase beta chain*	*sucC*	Q9JUT0	107, 109, 110	X	X	
Outer membrane protein class 4*	*rmpM*	P38367	50, 206, 207, 211, 279	X		X
Probable malate:quinone oxidoreductase	*mqo*	Q9JWK3	213, 214	X		
Glutaredoxin*		Q9JQS4	22, 23, 308, 310	X		
Porin B	*porB*	O53989	64, 247	X		
Dihydrolipoamide dehydrogenase*	*lpdA*	Q9JU06	86, 142		X	
L-lactate dehydrogenase*	*lldA*	Q9JTX1	115, 184			X
Putative succinate dehydrogenase flavoprotein subunit*	*sdhA*	Q9JUT3	125, 244	X		
Putative zinc-binding alcohol dehydrogenase*		Q9JVJ8	80, 311	X		
Transketolase	*tkt*	Q9JTR1	129, 137	X		
Putative polyribonucleotide nucleotransferase	*pnp*	Q9JV72	31, 234	X		
Serine hydroxymethyl transferase	*glyA*	Q9XAY7	1, 306	X		
Glutamine synthetase	*glnA*	Q9JSU6	15, 81	X		
ClpB protein*	*clpB*	Q9JTP9	30, 235			X
Putative formate-tetrahydrofolate ligase	*fhs*	Q9JVY8	35, 193	X		
Bifunctional purine biosynthesis protein purH	*purH*	Q9JUQ8	63, 194	X		
Putative cysteine synthase*	*cysK*	Q9JQL6	66, 113	X		
Putative dihydrolipoamide	*lpdA2*	Q9JUT5	69, 199	X		
Proline dehydrogenase	*putA*	Q9JSY1	36	X		
Putative 2-oxoglutarate dehydrogenase E1 component	*sucA*	Q9JRJ8	70	X		
Outer membrane protein Omp85	*omp85*	Q9JX31	68	X		
Probable D-lactase dehydrogenase	*dld*	Q9JUP8	134	X		
Pyruvate dehydrogenase E1 component	*aceE*	Q9JU08	127	X		
Glucosamine-fructose-6-phosphate aminotransferase	*glmS*	Q9JWN9	140	X		
5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	*metE*	Q9JUT6	148	X		

Table 3. Continued

Protein	Gene	Acc.	Spot or spot series	Type of isoform		
				Horizontal	Vertical	Diagonal
Putative phosphoenolpyruvate synthase	*ppsA*	Q9JV15	149	X		
Putative inosine-5'-monophosphate dehydrogenase	*guaB*	Q9JUD0	60	X		
Aconitate hydratase	*acnB*	Q9JTI5	231	X		
Putative isoleucyl-tRNA synthetase	*ileS*	Q9JVY4	232	X		
Leucyl-tRNA synthetase	*leuS*	Q9JW39	237	X		
DNA-directed RNA polymerase beta chain*	*rpoB*	P57009	301	X		
Carbamoyl phosphate synthase large chain	*carB*	Q9JW02	304	X		
Preprotein translocase SecA subunit*		Q9JYK8	305	X		

Horizontal and vertical indicate different types of spot patterns in which the M_r or pI values are constant, respectively. Diagonal indicates cases when significantly modified values for both M_r and pI were observed. The asterisks indicate proteins which are described as potential pathogenicity or virulence factors

predictions on biochemical pathways, based on genome analysis, is quite important since even within a species the gene content of a given pathway varies. For example, a phosphofructokinase isolated from *E. coli* was found to be absent from the published *E. coli* genome sequence [32]. In the map of strain Z4970 described here, we detected the expression of all the enzymes of the pyruvate dehydrogenase system. Interestingly, products of genes *aceE*, *lpdA*, *lpdA2*, *lpdA3* were present as multiple electrophoretic species, in agreement with the complexity of this metabolic system. With regards to TCA, the genome sequence of the Z2491 menA strain [20] showed the presence of a complete set of genes encoding the classic TCA cycle. In this study we found 10 of the 16 predicted TCA enzymes expressed; also in this case multiple electrophoretic species were found for almost all of them. Many glycolytic enzymes, including two electrophoretic species of glyceraldehyde-3-phosphate dehydrogenase C, were also detected in the map, in agreement with the fact that the bacteria we analyzed were grown on a glucose-based medium.

Amino acid biosynthesis was the third most abundant functional class among the proteins we identified. Although branched chain and aromatic families are represented, most of the identified enzymes are involved in serine, glutamate and aspartate biosynthesis. These three amino acids play a key role in the production of glycine and cysteine, glutamine, proline and arginine and asparagine, methionine, lysine, threonine and isoleucine, respectively. This probably implies a high activity in amino acid biosynthesis which is in agreement with the high levels of expression of the corresponding amino-acyl tRNA synthetases (see Section 3.3.2).

3.3.2 Macromolecule metabolism

Proteins belonging to the class synthesis and modification of macromolecules were the second most represented functional class, accounting for 36 proteins (multiple protein species excluded). We detected the expression of proteins involved in transcription, translation and protein modification, DNA replication, restriction/modification, recombination and repair. The most represented group within this functional class was constituted by 17 different amino-acyl tRNA synthetases. The massive presence of amino acyl tRNA synthetases correlates well both with the high presence of amino acid biosynthesis enzymes and the abundance of elongation factor Tu, which is involved in the binding and transport of aminoacyl-tRNA to the aminoacyl site of the ribosome. Although nothing has so far been reported for *Neisseria* on this topic, aminoacyl-tRNA synthetases also play an important regulatory role as translational repressors in prokaryotes by binding to their corresponding mRNA [33, 34].

The cell envelope was the fourth most abundant functional class represented in the map. Although the solubilization problems for membrane proteins are well known

limiting factors for protein resolution by 2-DE mapping, we could identify several periplasmic and membrane proteins, porins, proteins associated to surface structures such as pili, as well as proteins of the murein sacculus and peptidoglycan layer. It can be noted that superoxide dismutase (spot 268) catalyzes the conversion of the superoxide radical anion to hydrogen peroxide, a mechanism designed to preserve meningococci from the action of microbicidal oxygen-free radicals produced in the context of host defences [35].

Pili of pathogenic *Neisseriae* are typical of a family of adhesin, type IV fimbriae, found in a widerange of Gram-negative pathogens. They have a crucial role in both colonization of the host and adhesion to host cells [36]. Pili are composed of thousands of protein subunits, called pilins, which undergo both phase and antigenic variation and are post-translationally modified [37], and several regulators of pilus formation and retraction. Pilus retraction is an event required to mediate intimate adhesion of meningococci on the host-cell surface. We detected in these samples of bacteria grown on agar plates without specific environmental stimuli, three proteins involved in pilus assembly (pilF, pilO and pilP) and two forms of a protein controlling pilus retraction (pilT, and pilT2). Furthermore we also detected in the map the RegF protein, a pilE regulator. The menA capsule biosynthesis operon *sac* consists of four genes that are peculiar to serogroup A and are not found in other meningococcal serogroups. We observed in the Z4970 proteome the expression products of *sacA* and *sacB*, so far classified as putative, which are enzymes peculiar to serogroup A for *N*-acetylmannosamine-1-phosphate capsule biosynthesis [38].

3.4 Proteins potentially involved in meningococcal pathogenicity and virulence

Besides the main, generally acknowledged, pathogenicity factor of meningococci which is represented by the polysaccharide capsule, a number of meningococcal proteins have also been described as potential pathogenicity- and/or virulence-associated factors or products of genes which appear to be (up- or down-) regulated during virulence-associated events. Some of these are in fact among the proteins identified in the map we describe. However, the simple detection of expression of a given protein in bacteria grown in artificial laboratory conditions has only a limited significance, since the repertoire of genes required in various steps of pathogenic host invasion and disease development is likely to be induced (or, more generally, regulated) only in physiological relevant conditions, such as host-cell contact or interaction with molecules present in human serum. Considering that the preferred post-genomic technology for studying such events appears at present to be the study of transcript profiles using the microarray DNA hybridization approach rather than proteomic 2-DE mapping, we preferred to correlate our findings with results from microarray studies currently available in the scientific literature. The results of this analysis are reported in Table 4. It should be noted that some of the pathogenicity studies we refer to were actually performed on menigococci belonging to serogroup B. In these cases we entered the corresponding ortholog gene in Table 4, as annotated in the menA genome project [20], and the relative protein accession number in the Swiss-Prot/TrEMBL database. In particular, in Table 4 proteins are listed expressed by genes which are regulated following an interaction of the bacterium with a host-cell [39], proteins mediating adherence to epithelial cells [40, 41], proteins involved in heat shock, or better stress responses [42], and products of genes reported to be phase-variable [43].

3.4.1 Use of the map for comparative studies: Differential expression of transferrin-binding protein B in different genoclouds

Analyses of serogroup A meningococci isolated from epidemic waves have allowed the identification of nine clonal groupings, designated subgroups I-III, IV-1, IV-2 and V-VIII [44] and provided an opportunity to elucidate important features of bacterial microevolution. The genomes of bacterial isolates belonging to menA subgroup III, and which have caused two recent pandemic waves, were found to be very uniform, so that the comparatively few genetic variants which have been reported [44–46] were considered as mainly due to the import of genetic material of related species by DNA transformation during bacterial cocolonization of the nasopharynx [47, 48]. In 1987 menA subgroup III bacteria caused an outbreak in Mecca during the annual *Haj* pilgrimage [49], and after the return of pilgrims multiple meningococcal epidemics occurred throughout the African meningitis belt [7, 50]. This event provides a useful distinction between pre-Mecca and post-Mecca menA isolates. The study of the fit genotypes and variants within subgroup III, in pre- and post-Mecca epidemics, allowed Achtman and collaborators [10] to introduce the novel concept of genocloud, which can be defined as a frequent genotype plus its epidemiologically associated descendents, and to identify nine subgroup-III genoclouds comprising very closely related clinical isolates. Importantly, in subgroup III many of the genetic variants are escape variants that can evade the human immune system. A working hypothesis is that while some genoclouds extinguish after a single epidemic episode, others persist by migrating from

Table 4. Classification of identified proteins on the basis of reported pathogenicity, virulence and phase variability features

Spot	Protein name (SW/Tr)	Gene short name	Nb[a]	SW/Tr Accession no.[b]	Regulated by 16HB14 adhesion[c]	Expressed in serum-treated bacteria[d]	Regulated by Hep2 adhesion[e]	Regulated by HBMEC[f]	Heat-shock response[g]	PV[h]
22	Glutaredoxin		1141	Q9JQS4					X	
23	Glutaredoxin		1141	Q9JQS4					X	
30	ClpB protein	*clpB*	1683	Q9JTP9					X	
32	Putative maltose phosphorylase	*mapA*	2098	Q9JSW8	X					
39	Porin A	*porA*	1642	Q9JPT6		X	X			X
40	Porin A	*porA*	1642	Q9JPT6		X	X			X
50	Outer membrane protein class 4	*rmpM*	2105	P38367	X	X				
52	Glutamate dehydrogenase	*gdhA*	1964	Q9JT56	X			X		
53	ATP synthase alpha chain	*atpA*	0517	Q9JW72	X				X	
55b	Cysteine desulfurase	*iscS*	1594	Q9JTX0						X
56	Elongation factor G	*fusA*	0135	Q9JX07	X					
57	Elongation factor Tu	*tuf*	0134	Q9JRI5	X		X			
58	Electron transfer flavoprotein alpha-subunit	*etfA*	0241	Q9JWU3			X			
59	Elongation factor Ts	*tsf*	0327	Q9JRH4	X					
65	Succinyl-CoA synthetase alpha subunit	*sucD*	1154	Q9JUS9	X				X	
66	Putative cysteine synthase	*cysK*	0974	Q9JQL6	X					
67	ATP synthase beta chain	*atpD*	0519	Q9JW70	X					
69	Putative dihydrolipoamide dehydrogenase	*lpdA2*	1142	Q9JUT5					X	
78	Putative acetyl-CoA carboxylase biotin carboxylase component	*accC*	0596	Q9JW07			X			
80	Putative zinc-binding alcohol dehydrogenase		0808	Q9JVJ8					X	
84	Septum site-determining protein	*minD*	0100	Q9JQY6	X					
85	Oligopeptidase A	*prlC*	0054	Q9JX57	X				X	
87	Putative GTP-binding protein	*typA*	1370	Q9JUD2	X					
91a	Trigger factor	*tig*	1526	Q9JU32	X	X				
103	Phosphoglycerate kinase	*pgk*	0257	Q9JWS8	X					
106	DNA-directed RNA polymerase alpha subunit	*rpoA*	0103	Q9JR06	X					
107	Succinil-CoA synthetase beta chain	*sucC*	1153	Q9JUT0	X					
108	Dihydrolipoamide putative succinyltransferase E2 component	*sucB*	1150	Q9JUT2	X					
109	Succinyl-CoA synthetase beta chain	*sucC*	1153	Q9JUT0	X					
110	Succinyl-CoA synthetase beta chain	*sucC*	1153	Q9JUT0	X					
112	Adenylosuccinate synthetase	*purA*	1024	Q9JV25	X					
113	Putative cysteine synthase	*cysK*	0974	Q9JQL6	X					
114	Putative pilus retraction protein	*pilT2*	0979	Q9JV63		X			X	

Table 4. Continued

Spot	Protein name (SW/Tr)	Gene short name	Nb[a]	SW/Tr Accession no.[b]	Regulated by 16HB14 adhesion[c]	Expressed in serum-treated bacteria[d]	Regulated by Hep2 adhesion[e]	Regulated by HBMEC[e]	Heat-shock response[f]	PV[g]
115	L-lactate dehydrogenase	*lldA*	1592	Q9JTX1			X		X	X
121	Chaperone protein dnaK	*dnaK*	0736	Q9JVQ9	X				X	
123	Putative aminopeptidase		1640	Q9JTT6	X					
124	Pyruvate kinase	*pykA*	0177	Q9JWX8	X					
125	Putative succinate dehydrogenase flavoprotein subunit	*sdhA*	1145	Q9JUT3				X		
126	Acetolactate synthase isozyme III large subunit	*ilv1*	1766	Q9JTI1			X	X		
135	60 KDa chaperonin	*groEL*	0473	P57006	X				X	
142	Dihydrolipoamide dehydrogenase	*lpdA*	1556	Q9JU06	X					
147	Cell division protein ftsZ	*ftsZ*	2057	Q51130	X					
155	Probable sulphate adenylate transferase subunit 1	*cysN*	1364	Q9JUD7	X					
158	Putative oxidoreductase		0666	Q9JVV3					X	
160	2,3,4,5-tetrahydropyridine-2-carboxylate *N*-succinyltransferase	*dapD*	2153	Q9JSS7	X					
161a	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	X			X		
162	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	X			X		
163	Glutamate dehydrogenase	*gdhA*	1964	Q9JT56	X			X		
165	Ketol-acid reductoisomerase	*ilcV*	1763	Q9JTI3			X	X		
169a	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	X			X		
170	Putative alcohol dehydrogenase	*adhA*	0725	Q9JVR8	X			X		
176a	Putative membrane transport solute-binding protein	*fetB*	0452	Q9JWB9		X				
180	S-adenosylmethionine synthetase	*metK*	0663	Q9JVV6	X					
182	Ribose-phosphate pyrophosphokinase	*prs/prsA*	1093	Q9JQV4	X					
184	L-lactate dehydrogenase	*lldA*	1592	Q9JTX1			X		X	
187	Delta-aminolevulinic acid dehydratase	*hemB*	1011	Q9JV37	X					
189	30S Ribosomal protein S2	*rpsB*	0328	Q9JRG7	X					
195	Argininosuccinate lyase	*argH*	0847	Q9JVG7	X					
197	Hypothetical protein NMA0866		0866	Q9JVF0	X					
199	Putative dihydrolipoamide dehydrogenase	*lpdA2*	1142	Q9JUT5					X	
203	Lactoferrin-binding protein	*lbpB*	1740	Q9JTK3			X			
204	Adenylate kinase	*adk*	1032	P49980	X					
206	Outer membrane protein class 4	*rmpM*	2105	P38367	X	X				
207	Outer membrane protein class 4	*rmpM*	2105	P38367	X	X				

Table 4. Continued

Spot	Protein name (SW/Tr)	Gene short name	Nb[a]	SW/Tr Accession no.[b]	Regulated by 16HB14 adhesion[c]	Expressed in serum-treated bacteria[d]	Regulated by Hep2 adhesion[e]	Regulated by HBMEC[e]	Heat-shock response[f]	PV[g]
210	Glutamate-1-semialdheyde 2,1-aminomutase	*hemL*	0592	Q9JW10	X					
211	Outer membrane class 4 protein	*rmpM*	2105	P38367	X	X				
212	Ribose-phosphate pyrophosphokinase	*prs/prsA*	1093	Q9JQV4	X					
215	DNA helicase II	*uvrD*	0027	Q9JR27				X		
221	Pilus assembly protein	*pilO*	0652	Q9JR13		X	X		X	
227	Transcription antitermination protein NusG	*nusG*	0147	Q9JRD9			X			
230	Glyceraldehyde-3-phosphate dehydrogenase	*gapA*	0062	Q9JX51	X					
235	clpB protein	*clpB*	1683	Q9JTP9					X	
241	Glutamate dehydrogenase	*gdhA*	1964	Q9JT56	X			X		
244	Putative succinate dehydrogenase flavoprotein subunit	*sdhA*	1145	Q9JUT3				X		
245	Transcription termination factor	*rho*	0825	Q9JVI6	X		X			
246	33kDa chaperonin	*hslO*	0441	Q9JWC8			X			
254	Pilus assembly protein	*pilP*	0651	Q9JQN6	X	X			X	
258	Peptidyl-prolyl cis-trans isomerase B	*ppiB*	1002	Q9JQS5	X				X	
259	ATP synthase delta chain	*atpH*	0516	Q9JW73	X				X	
261	Hypothetical protein NMA1203		1203	Q9JUP9	X		X			
266	Triosephosphate isomerase	*tpiA*	0570	Q9JW31			X			
267	ATP-dependent Clp protease proteolytic subunit	*clpP*	1525	Q9JU33	X					
268	Hypothetical protein NMA2195	*ycl026C -A*	2195	Q9JQW5	X					
270	Dihydrodipicolinate reductase	*dapB*	0066	Q9JX48	X					
273	Orotate phosphoribosyl-transferase	*pyrE*	0582	Q9JR25	X					
277	Adhesin	*mafA*	0325	Q9JWK7	X					X
279	Outer membrane protein class 4	*rmpM*	2105	P38367	X	X				
282	Methionine aminopeptidase	*map*	0337	Q9JWK1						X
283	Putative gntR-family transcriptional regulator		1751	Q9JRE6					X	
291	Putative marR-family transcriptional regulator		0613	Q9JR77				X		
298	3-oxoacyl-[acyl-carrier-protein] syntase III	*fabH*	0538	Q9JW56	X					
300	Amino acid ABC transporter, ATP-binding protein		0900	Q9JVC3		X			X	
301	DNA-directed RNA polymerase beta chain	*rpoB*	0142	P57009			X			

Table 4. Continued

Spot	Protein name (SW/Tr)	Gene short name	Nb[a]	SW/Tr Accession no.[b]	Regulated by 16HB14 adhesion[c]	Expressed in serum-treated bacteria[d]	Regulated by Hep2 adhesion[e]	Regulated by HBMEC[f]	Heat-shock response[g]	PV[g]
305	Preprotein translocase SecA subunit		1536	Q9JYK8	X					
307	Putative amino acid permease substrate-binding protein		0997	Q9JV46	X	X	X		X	
308	Glutaredoxin		1141	Q9JQS4					X	
310	Glutaredoxin		1141	Q9JQS4					X	
311	Putative zinc-binding alcohol dehydrogenase		0808	Q9JVJ8					X	
312	UDP-*N*-acetylmuramate-L-alanine ligase	*murC*	2061	Q8JSZ8	X					

a) Gene numbers (NMAxxxx) according to [20] and the menA database at the NCBI web site at http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html. When the quoted literature data referred to a serogroup B strain the corresponding menA orthologous genes were identified by BLAST homology searches
b) Swiss-Prot/TrEMBL accession number
c) Grifantini R. *et al.* [39]
d) Kurtz S. *et al.* [40]
e) Dietrich G. *et al.* [41]
f) Guckenberger M. *et al.* [42]
g) PV = Phase Variability. Snyder, L. A. S. *et al.* [43]

country to country and by generating new genetic variants that escape human immune response against previous infections [10].

Pathogenic *Neisseria* have a siderophore-independent iron-uptake system to overcome the iron-restricted composition of host extracellular fluids. This outer membrane system consist of two subunits, transferrin-binding proteins A and B (TbpA and TbpB, respectively). They form a receptor which captures the iron-carrier human transferrin [51] in order to provide the bacterium with Fe ions. The TbpB subunit is largely external to the outer membrane, has an *N*-terminal lipid anchor and, being a target for host immune responses, is also considered a potential vaccine candidate [52]. Both TbpB antigenic and genetic variability have been observed, the latter being due to the occurrence of horizontal genetic exchanges between strains as well as to intragenic recombination [53]. TbpB is encoded by the *tbpB* polymorphic locus, which has been used to study by sequence typing the molecular epidemiology and bacterial microevolution of menA. This approach was instrumental for the definition of the genocloud concept [10]. Achtman and colleagues described 28 *tbpB* alleles; strain Z4970, analyzed in the present paper, belongs to subgroup III, genocloud 3 and contains *tpbB1*, an ancestral allele of older strains of subgroups III and IV-1 (Fig. 5) [10].

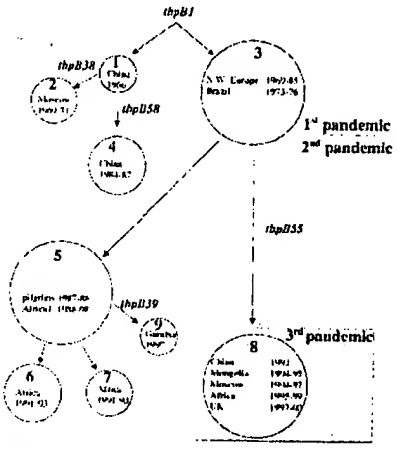


Figure 5. Variation of *tbpB* alleles in nine subgroup III genoclouds. Numbered circles represent the individual genoclouds and there sizes are proportional to the number of menA isolates. The figure has been simplified from [10].

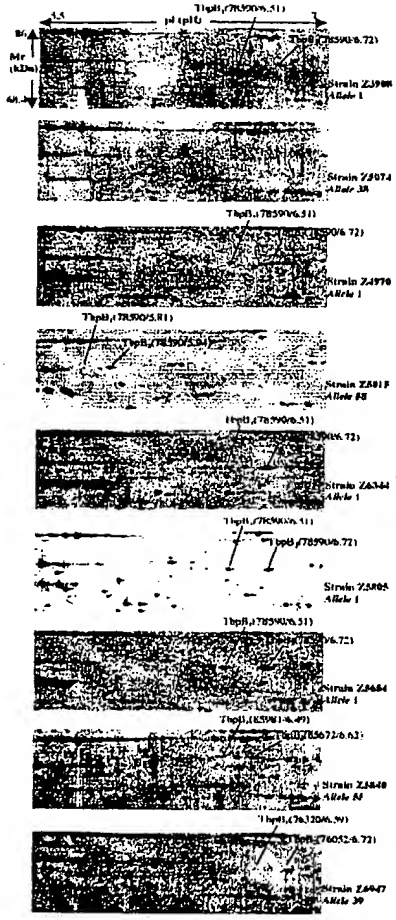


Figure 6. Mapping of TbpB in different strains of *N. meningitidis* serogroup A, subgroup III. Each strain (identified by the ID code on the right of each image section) belongs to a different genocloud as summarized in Table 5. The gel areas shown cover the same p*I*/*M*$_r$ ranges of silver-stained 2-DE gels. Experimental p*I*/*M*$_r$ coordinates of TBpB spots in each single strain are indicated in brackets, as well as the identification number of the *tbpB* alleles assigned to each genocloud in [10].

Considering the reported low grade of genetic variation in menA clonal populations, one could reasonably expect that 2-DE protein maps of menA clinical isolates should be sufficiently similar to allow comparative studies aiming at identifying minor phenotypic variations between genoclouds by simple *in silico* comparison against a fully MS-annotated reference map. To test this hypothesis we compared the proteomes of nine menA subgroup-III strains, each one being a representative of one of the nine diverse genoclouds described in [16] (Fig. 6 and Table 5). The proteome maps of these 9 strains were more than 95% identical. Figure 6 shows the different patterns with which two TbpB protein species are present in each strain. For the Z4970 map the TbpB spots were identified by MS analysis, as above described. For the Z5015 map additional MS analyses were performed (data not shown) and the identification of the two acidic species of TbpB was also obtained by MALDI-TOF MS analysis. For the remaining seven maps, TbpB spots were identified by *in silico* image matching against the Z4970 and Z5015 maps. In doing so, the large set of invariant spots was used as local matching anchors. Visual recognition of local spot-patterns, within set tolerance limits for M_r/p*I*, was used to assign the TbpB spot identity to spots in the Z5840 and Z6947 maps. For strain Z5074 we could not identify TbpB spots by simple map matching, maybe because of a low level of expression in this particular strain. However, this anyway indicated an individual peculiarity of Z5074 with respect to the other genocloud strains. The results of the experiment, summarized in Table 5, showed that the relative diversity of the p*I*/*M*$_r$ coordinates, or better, the diverse types of electrophoretic phenotypes (map patterns) observed for TbpB in the 9 genocloud maps, are in complete agreement with genocloud classification of the isolates as described by Achtman and collaborators [10] on the basis of genotyping analyses.

4 Concluding remarks

In this study, we report the first systematic proteomic analysis of a serogroup A *N. meningitidis* strain, which is expected to provide a basis for more extensive proteomic studies addressing meningococcal biology. Also, we confirmed the expected very low variability of the menA proteome, a feature which should favor comparative studies. As a proof of principle for such an application, we performed a comparative analysis on the B-subunit of the meningococcal transferrin receptor, a known marker of population diversity in meningococci. The results showed that TbpB spot pattern variation, as observed in the maps of nine clinical isolates from diverse epidemic spreads, fits previous analyses based on allelic variations of the *tbpB* gene. This exercise showed that proteomic pheno-

Table 5. Features of *N. meningitidis* serogroup A subgroup III strains, representative of the nine genoclouds, analyzed in Fig. 6

Strain	Geno-cloud	Isolate	Pandemic wave	*tbp*B allele	2-DE pattern	pI Theor	pI Exp
Z3908	1	Ancestral	1st	*tbp*B1	1	6.49	6.51/6.72
Z5074	2	Moscow 69–71	1st	*tbp*B38	0 (nd)	6.28	(nd)
Z4970	3	Ancestral	1st	*tbp*B1	1	6.49	6.51/6.72
Z5015	4	China 84–87	2nd	*tbp*B58	2	5.77	5.81/5.94
Z6344	5	Post-Mecca	2nd	*tbp*B1	1	6.49	6.51/6.72
Z5805	6	Post-Mecca	2nd	*tbp*B1	1	6.49	6.51/6.72
Z5654	7	Post-Mecca	2nd	*tbp*B1	1	6.49	6.51/6.72
Z5840	8	China	3rd	*tbp*B55	3	6.40	6.49/6.62
Z6947	9	Post-Mecca	2nd	*tbp*B39	4	7.27	6.59/6.72

Clinical and epidemiological annotation are reported from [10]. pI Theor, theoretical pI values deduced from published nucleotide sequences of *tbpB* alleles expected to be prevalent in the corresponding genoclouds reported in column 2. The table shows that the relative variations of pIs in columns 7 and 8 are internally consistent. The different electrophoretic TbpB spot patterns in Fig. 6 are identified by arbitrary numbers. nd: not determined

type analysis could be useful for identifying new markers of menA microevolution. In fact preliminary systematic comparisons with the reference menA map described here and data (not shown), when extended to the entire map of the nine genocloud isolates, identified some 15 spot patterns so far suggesting that other protein species undergo significant phenotypic variations in different genoclouds. Future work will require further MS analyses and comparative gene sequencing in order to find out if these genes can actually be proposed as new microevolution markers.

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5 References

[1] Hubert, B., Caugant, D. A., *Eurosurveillance* 1997, *2*, 69–71.
[2] WHO, *Control of Epidemic Meningococcal Disease*, 2nd edition, *WHO Practical Guidelines*, Geneva 1998.
[3] Nassif, X., Bourdoulous, S., Eugene, E., Couraud, P. O., *Trends Microbiol.* 2002, *10*, 227–232.
[4] Taha, M. K., Deghmane, A. E., Antignac, A., Zarantonelli, M. L. *et al.*, *Trends Microbiol.* 2002, *10*, 376–382.
[5] Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Popovic, T., Hughes, J. M., *N. Engl. J. Med.* 2001, *344*, 1378–1388.
[6] Vedros, N. A., *Development of Meningococcal Serogroups in Evolution of Meningococcal Disease*, vol. II, CRC Press, Boca Raton, FL, USA 1987.
[7] Achtman, M., in: Cartwright, K. (Ed.), *Global Epidemiology of Meningococcal Disease in Meningococcal Disease*, John Wiley, Chichester, England 1995, pp. 159–175.
[8] Norais, N., Nogarotto, R., Iacobini, E. T., Garaguso, I. *et al.*, *Proteomics* 2001, *1*, 1378–1389.
[9] http://www.abdn.ac.uk/~mmb023/neisseria/neist5_f.htm.
[10] Zhu, P., van der Ende, A., Falush, D., Brieske, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 2001, *98*, 5234–5239.
[11] Chevallet, M., Santoni, V., Poinas, A., Rouquie, D. *et al.*, *Electrophoresis* 1998, *11*, 1901–1909.
[12] Bradford, M. M., *Anal. Biochem.* 1976, *7*, 248–254.
[13] Hochstrasser, D. F., Patchornik, A., Merril, C. R., *Anal. Biochem.* 1988, *173*, 412–423.
[14] Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., *Electrophoresis* 1988, *9*, 255–262.
[15] Zhang, W., Chait, B.T., *Anal. Chem.* 2000, *72*, 2482–2489.
[16] Clauser, K. R., Baker, P. R., Burlingame, A. L., *Anal. Chem.* 1999, *71*, 2871–2882.
[17] Santucci, A., Trabalzini, L., Bovalini, L., Ferro, E. *et al.*, *Electrophoresis* 2000, *21*, 3717–3123.
[18] Trabalzini, L., Paffetti, A., Scaloni, A., Talamo, F. *et al.*, *Biochem. J.* 2003, *370*, 35–46.
[19] Gardy, J. L., Spencer, C., Wang, K., Ester, M. *et al.*, *Nucleic Acids Res.* 2003, *31*, 3613–3617.
[20] Parkhill, J., Achtman, M., James, K. D., Bentley, S. D. *et al.*, *Nature* 2000, *404*, 502–506.
[21] Bjellqvist, B., Basse, B., Olsen, E., Celis, J. E., *Electrophoresis* 1994, *15*, 529–539.
[22] Vandhal, B. B., Birkelund, S., Demol, H., Hoorelbeke, B. *et al.*, *Electrophoresis* 2001, *22*, 1024–1223.
[23] Celdas, T. D., Yaagoubi, E., Richarme, G., *J. Biol. Chem.* 1988, *273*, 11478–11482.

[24] Klugman, K. P., Gotschlich, E. C., Blake, M. S., *Infect. Immun.* 1989, *57*, 2066–2071.

[25] Gotschlich, E. C., Seiff, M., Blake, M. S., *J. Exp. Med.* 1987, *165*, 471–482.

[26] Williams, K. R., Hemmings, H. C. Jr., LoPresti, M. B., Konigsberg, W. H., Greengard, P., *J. Biol. Chem.* 1986, *261*, 1890–1903.

[27] Tozawa, K., Broadhurst R. W., Raine, A. R. C.,Fuller, C. *et al.*, *Eur. J. Biochem.* 2001, *268*, 4908–4917.

[28] Sankaran, K., Wu, H. C., *J. Biol. Chem.* 1994, *269*, 19701–19706.

[29] Petit, C. M., Brown, J. R., Ingraham, K., Bryant A. P., Holmes, D. J., *FEMS Microbiol. Lett.* 2001, *200*, 229–233.

[30] Schlee, S., Beinker, P., Akhrymuk, A., Reinstein, J., *J. Mol. Biol.* 2004, *336*, 275–285.

[31] Leighton, M. P., Kelly, D. J., Williamson, M. P., Shaw, J. G., *Microbiology* 2001, *147*, 1473–1482.

[32] Huynen, M. A., Dandekar, T., Bork, P., *Trends Microbiol.* 1999, *7*, 281–291.

[33] Schlax, P. J., Worhunsky, D. J., *Mol. Microbiol.* 2003, *48*, 1157–1169.

[34] Romby, P., Springer, M., *Trends Genet.* 2003, *19*, 155–161.

[35] Wilks, K. E., Dunn, K. L., Farrant, J. L., Reddin, K. M. *et al.*, *Infect. Immun.* 1998, *66*, 213–217.

[36] Marceau, M., Nassif, X., *J. Bacteriol.* 1999, *181*, 656–661.

[37] Virji, M., Saunders, J. R., Sims, G., Makepeace, K. *et al.*, *Mol. Microbiol.* 1993, *10*, 1013–1028.

[38] Dolan-Livengood, J. M., Miller, Y. K., Martin, L. E., Urwin, R. *et al.*, *J. Infect. Dis.* 2003, *187*, 1616–1628.

[39] Grifantini, R., Bartolini, E., Muzzi, A., Draghi, M. *et al.*, *Nat. Biotechnol.* 2002, *20*, 914–921.

[40] Kurz, S., Hubner, C., Aepinus, C., Theiss, S. *et al.*, *Vaccine* 2003, *21*, 768–775.

[41] Dietrich, G., Kurz, S., Hubner, C., Aepinus, C. *et al.*, *J. Bacteriol.* 2003, *185*, 155–164.

[42] Guckenberger, M., Kurz, S., Aepinus, C., Theiss, S. *et al.*, *J. Bacteriol.* 2002, *184*, 2546–2551.

[43] Snyder, L. A. S., Butcher, S. A., Saunders, N. J., *Microbiology* 2001, *147*, 2321–2332.

[44] Wang, J. F., Caugant, D. A., Li, X., Hu, X. *et al.*, *Infect. Immun.* 1992, *60*, 5267–5282.

[45] Achtman, M., Kusecek, B., Morelli, G., Eickmann, K. *et al.*, *J. Infect. Dis.* 1992, *165*, 53–68.

[46] Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G. *et al.*, *Proc. Natl. Acad. Sci. USA* 1998, *95*, 3140–3145.

[47] Linz, B., Schenker, M., Zhu, P., Achtman, M., *Mol. Microbiol.* 2000, *36*, 1049–1058.

[48] Smith, J. M., Feil, E. J., Smith, N. H., *BioEssays* 2000, *22*, 1115–1122.

[49] Moore, P. S., Harrison, L. H., Telzak, E. F., Ajello, G. W., Broome, C. V., *J. Am. Med. Assoc.* 1988, *260*, 2686–2689.

[50] Morelli, G., Malorny, B., Muller, K., Seiler, A. *et al.*, *Mol. Microbiol.* 1997, *25*, 1047–1064.

[51] Evans, R. W., Oakhill, J. S., *Biochem. Soc. Trans.* 2002, *30*, 705–707.

[52] Rokbi, B., Renauld-Mongenie, G., Mignon, M., Danve, B. *et al.*, *Infect. Immun.* 2000, *68*, 4938–4947.

[53] Legrain, M., Rokbi, B., Villeval, D., Jacobs E., *Gene* 1998, *208*, 51–59.

BOEHRINGER MANNHEIM

S. Rioux

The DIG System User's Guide for Filter Hybridization

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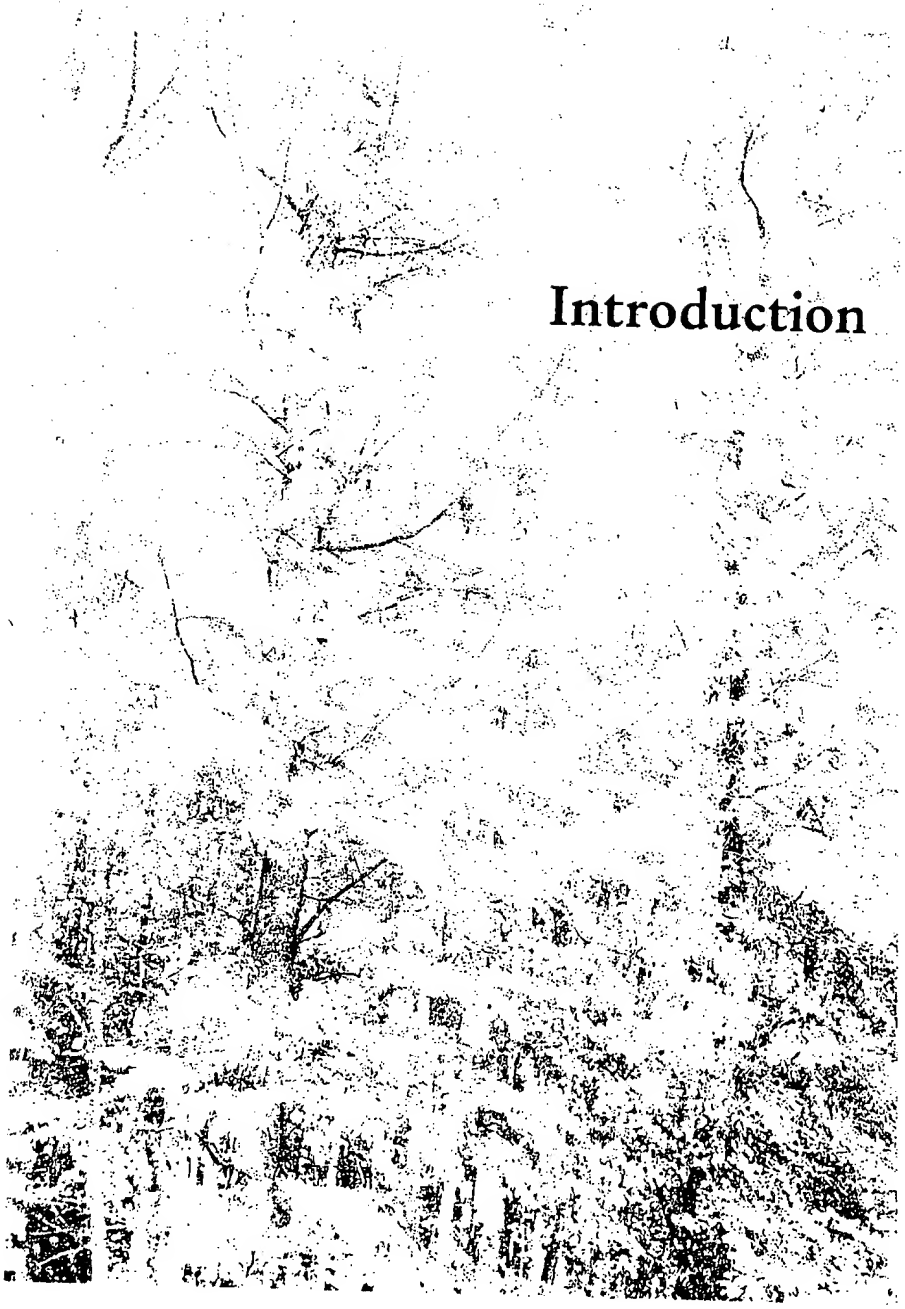
The DIG System User's Guide for Filter Hybridization

Contents



Introduction

Introduction

Welcome to the DIG Nonradioactive Nucleic Acid Labeling and Detection System

The DIG System is the most comprehensive, convenient, and effective system for the labeling and detection of DNA, RNA, and oligonucleotides. It also does not require you to learn any new technology. The protocols for labeling with digoxigenin and subsequent detection are based on well-established, widely used methods. DNA, RNA, and oligonucleotide probes are labeled according to the methods (usually enzymatic) used for preparing radioactive probes. Hybridization of digoxigenin-labeled probes (e.g., to target DNA or RNA on a Southern or Northern blot) is also carried out according to standard protocols, except that a special blocking reagent is used to eliminate background. The signal on the nucleic acid blot is detected according to the methods developed for western blots. An anti-digoxigenin antibody-alkaline phosphatase conjugate is allowed to bind to the hybridized probe. The signal is then detected with colorimetric or chemiluminescent alkaline phosphatase substrates. If a colorimetric substrate is used, the signal develops directly on the membrane. The signal is detected on an X-ray film (as with ^{32}P or ^{35}S-labeled probes) when a chemiluminescent substrate is used.

DIG Labeling

The use of a nonradioactive probe confers several advantages:

- The technology is safe.
- Probes can be stored for at least a year.
- Hybridization solutions can be reused several times.

The incorporation and spacing of digoxigenin (Figure 1) in DNA, RNA, and oligonucleotides has been optimized for the greatest sensitivity in Boehringer Mannheim's Kits for nucleic acid labeling:

- PCR DIG Probe Synthesis Kit – Digoxigenin-11-dUTP is incorporated by the polymerase chain reaction.
- DIG DNA Labeling and Detection Kit and the DIG DNA Labeling Kit – Digoxigenin-11-dUTP is incorporated by the random-primed labeling method.
- DIG RNA Labeling Kit – The Kit uses T7/SP6-mediated transcription for the synthesis of strand-specific RNA probes.
- DIG Oligonucleotide 3'-End Labeling Kit – Terminal transferase adds a single Digoxigenin-11-ddUTP to the 3'-end of the oligonucleotide.
- DIG Oligonucleotide Tailing Kit – Terminal transferase adds a string of Digoxigenin-11-dUTP interspersed with unlabeled dATP to the 3'-end of oligonucleotides.
- DIG Oligonucleotide 5'-End Labeling Set – DIG-NHS ester labels the 5'end.

In addition, protocols have been optimized for nick translation and cDNA synthesis.

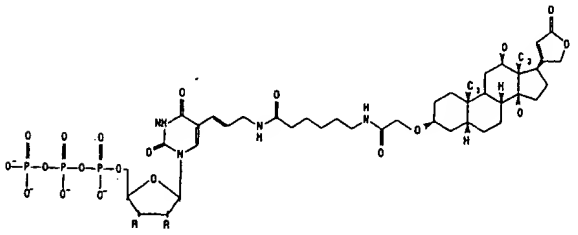


Figure 1: General Structure of DIG-labeled nucleotides. Alkali-stable Digoxigenin-UTP (R_1 = OH, R_2 = OH); Digoxigenin-dUTP (R_1 = OH, R_2 = H); Digoxigenin-ddUTP (R_1 = H, R_2 = H);

As in experiments that use radioactive probes, the yield of the labeling reaction should be estimated to ensure the success of the reaction and to approximate the amount of probe to be used in the hybridization experiment. A simple dot blot method is used to estimate probe yield; the protocol can be found on page 33. Before hybridization, we also recommend that the optimal probe concentration be determined with a "mock hybridization", where various amounts of probe in hybridization solution are hybridized to naked pieces of membrane. This brief procedure ensures high sensitivity and avoids the possibility of high background attributable to a probe concentration that is too high (see page 42).

DIG Detection

Several alternatives are available for the detection of digoxigenin-labeled probes.

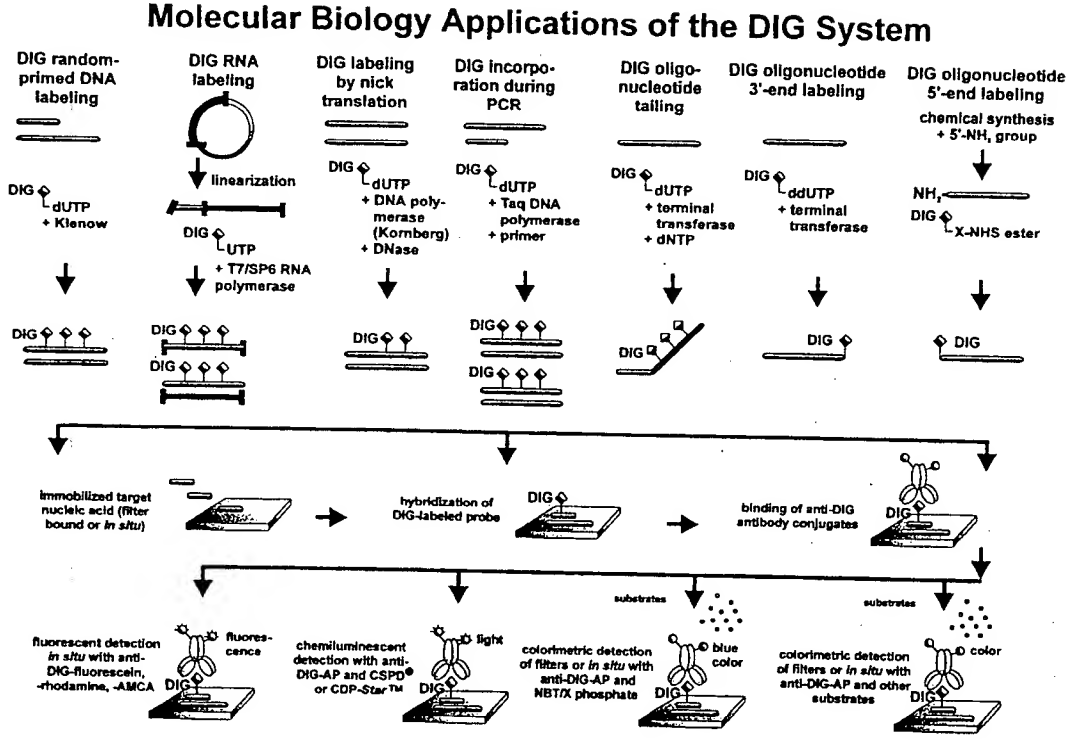
- DIG Luminescent Detection Kit for Nucleic Acids – Uses the chemiluminescent alkaline substrate CSPD® to produce a light signal, which is detected by exposing the membrane to an X-ray film.
- DIG Nucleic Acid Detection Kit – Uses the colorimetric substrates NBT and BCIP to generate purple/brown precipitate directly on the membrane.
- Multicolor Detection Set – The set comprises three naphthol-AS-phosphate/diazonium salt combinations for the visualization of a green, red, or blue hybridization signal; the dyes are used in combination with the DIG Nucleic Acid Detection Kit or the corresponding single reagents.

There is also a wide range of alternative anti-digoxigenin conjugates available, such as Anti-Digoxigenin-Peroxidase, Anti-Digoxigenin-Gold, Anti-Digoxigenin-Fluorescein, Anti-Digoxigenin-Rhodamine, Anti-Digoxigenin-AMCA. See Appendix C for a complete listing.

The Power of the DIG System

The DIG Nonradioactive Nucleic Acid Labeling and Detection System can be used for single-copy gene detection on human genomic Southern blots, the detection of unique mRNA species on Northern blots, colony and plaque screening, dot/slot blots, and *in situ* hybridization. Examples and protocols for these applications (except for *in situ* hybridizations) can be found throughout the *DIG System User's Guide*. See Figure 2 for an overview of DIG System labeling and detection alternatives. For a comprehensive treatment of nonradioactive *in situ* hybridization, ask for a free copy of Boehringer Mannheim's "Nonradioactive *In Situ* Hybridization Manual". The DIG System can also be used for nonradioactive sequencing; see page 70.

Figure 2: The labeling and detection alternatives offered by the DIG System.



An Overview of the DIG System User's Guide

Intent of the User's Guide

This manual describes all the digoxigenin labeling methods and all the alkaline phosphatase-based detection assays. All labeling and detection methods are presented in one manual so that users of the DIG System have a convenient reference on which to base their experiments.

What's New About this Version of the User's Guide

This version of the *DIG System User's Guide for Filter Hybridization* includes expanded procedures for labeling DNA probes with DIG by PCR. In addition, it includes many newly released products that increase the convenience of the DIG System, such as the DIG Oligonucleotide 5'-End Labeling Set, DIG Easy Hyb, and the DIG Wash and Block Buffer Set.

How to Use the User's Guide

To use the User's Guide, simply select one of the stand-alone sections from each division, choosing one labeling method, one hybridization technique, and one detection method. If you have not already done so, you can then order the required DIG kits or individual DIG System components (listed in the "Required products" table) by consulting Appendix C for complete ordering information. Upon receiving a DIG kit, immediately refer to Appendix A, which contains a complete listing of each kit's components. Use the following flow chart to perform nonradioactive nucleic acid labeling and detection.

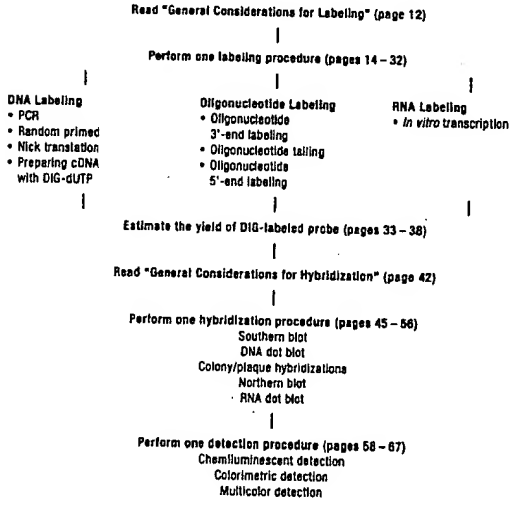
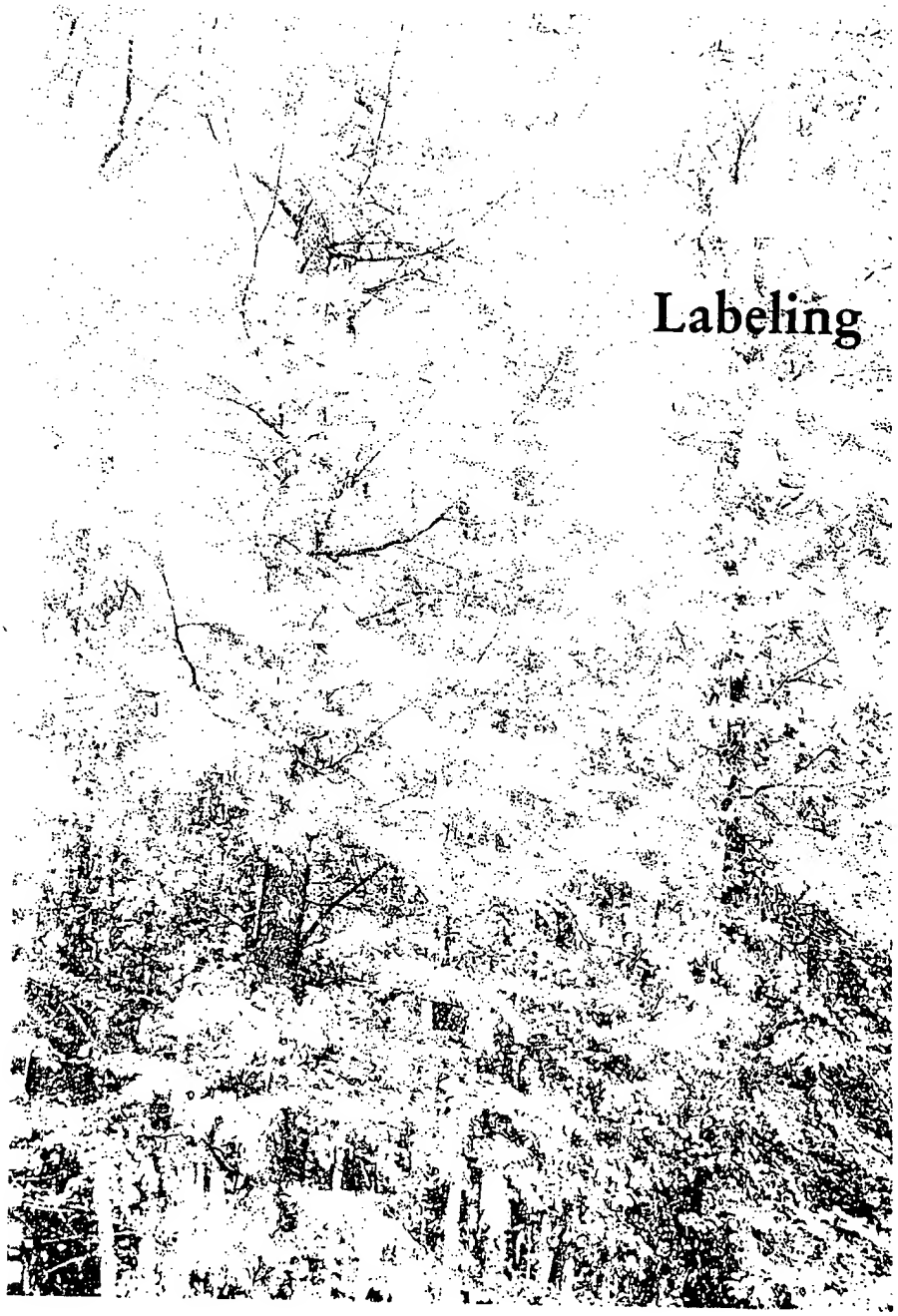


Figure 3: Flow chart for using the *DIG System User's Guide for Filter Hybridization*



Labeling

Template Purity

In general, the higher the purity of the DNA template, the better the labeling efficiency. We routinely phenol: $CHCl_3$ extract our DNA templates prior to the labeling reaction. In addition, for the random primed DNA labeling method, it is critical that you linearize and heat-denature the template prior to the labeling reaction.

Oligonucleotides should be gel purified or HPLC purified prior to 3'-end labeling or 3'-tailing.

Labeling Procedures

When using the DIG System, DNA probes can be labeled by a number of methods. RNA probes are labeled by *in vitro* transcription. Oligonucleotides are labeled by 3'-end labeling, 3'-tailing, or 5'-end labeling.

The choice of probe labeling method will be dependent on the following factors:

- The application you have; e.g. are you performing Northern blotting or Southern blotting.
- The template you have available for probe preparation; e.g. cloned insert or oligonucleotide.
- The sensitivity that must be achieved; e.g. single copy gene detection or detection of amplified DNA fragments.

Table 1 lists the most suited labeling methods for the different applications, with an indication of the sensitivity that can be achieved.

Table 1 ▶

Application	Labeling Method	Relative Sensitivity
Southern blotting	Random primed DNA labeling	+++
	Incorporation of DIG-11-dUTP during PCR	+++
	Labeling RNA by *in vitro* transcription	+++
	3'-Tailing of oligonucleotides	++
	3'- and/or 5'-End labeling of oligonucleotides	+
Northern blotting	Labeling RNA by *in vitro* transcription	++++
	3'-Tailing of oligonucleotides	++
	3'- and/or 5'-End labeling of oligonucleotides	+
	Random primed DNA labeling	++[1]
	Incorporation of DIG-11-dUTP during PCR	++[1]
Dot-/slot blotting	Random primed DNA labeling	+++
	3'-Tailing of oligonucleotides	+++
	3'-End labeling of oligonucleotides	++
	Labeling RNA by *in vitro* transcription	+++
	Incorporation of DIG-11-dUTP during PCR	+++
Colony/plaque hybridization	Random primed DNA labeling	+++[2]
	3'- and/or 5'-End labeling of oligonucleotides	+++
	Incorporation of DIG-11-dUTP during PCR	+++[2]
Sequencing	5'-End labeling of oligonucleotides	++
In Situ Hybridization	Nick translation	++
	Labeling RNA by *in vitro* transcription	+++
	3'-Tailing of oligonucleotides	++
	Incorporation of DIG-11-dUTP during PCR	+++

1 DNA probes cannot be recommended for Northern blotting, and should only be used when no other possibility remains.
2 Care must be taken that probes obtained from plasmids contain no vector sequences.

Assay of DIG-labeled Probes

It is important to check the efficiency of each labeling reaction. The purpose of this is to

- confirm the success of the labeling reaction
- estimate the yield of DIG-labeled probe, which must be known for the subsequent hybridization step.

Probe assay procedures are easy to perform and are described in the section entitled "Estimating the Yield of DIG-labeled Nucleic Acids", which begins on page 33. DIG-labeled controls provided in the DIG kits or sold separately are required for these probe-estimation assays. The estimation of probe yield can also conveniently be performed with the DIG Quantification Teststrips and DIG Control Teststrips.

Storage of DIG-labeled Probes

One of the major advantages of the DIG System is the long-term stability of DIG-labeled probes. DIG-labeled DNA probe solutions can be stored at –20°C (DIG-labeled RNA probe solutions should be stored at –70°C) for at least 1 year without loss of activity.

Incorporation of Digoxigenin-11-dUTP During PCR

Digoxigenin-11-dUTP (DIG-dUTP) can be incorporated by Taq DNA Polymerase during polymerase chain reactions. The resulting probes are very sensitive, and the yield from the labeling reaction is quite high.

Generation of DIG-labeled Probes with the PCR DIG Probe Synthesis Kit*

The PCR DIG Probe Synthesis Kit (Cat. No. 1 636 090) provides maximum convenience in generating DIG-labeled probes by PCR. This kit's ready-to-use PCR DIG Probe Synthesis Mix features a 1:2 DIG-11-dUTP:dTTP ratio. This ratio can be used to produce probes for a wide range of filter hybridization applications. Probes generated with this kit can be used to detect single-copy genes in genomic Southern blotting procedures.

Products required

Required product	Description	Available as
PCR buffer without $MgCl_2$	100 mM Tris-HCl; 500 mM KCl; pH 8.3 (20°C)	• Vial 3, PCR DIG Probe Synthesis Kit
$MgCl_2$ stock solution	25 mM $MgCl_2$	• Vial 4, PCR DIG Probe Synthesis Kit
PCR DIG Probe Synthesis Mix	2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP, 0.7 mM alkali-labile DIG-11-dUTP, pH 7.0	• Vial 2, PCR DIG Probe Synthesis Kit
Taq DNA Polymerase	5 U/µl Taq DNA Polymerase	• Vial 1, PCR DIG Probe Synthesis Kit
Control template (for control reactions only)	20 pg/µl plasmid DNA in Tris/EDTA buffer, pH 8.0 The 5 kb plasmid contains the cDNA for human tissue type plasminogen activator (tPA).	• Vial 5, PCR DIG Probe Synthesis Kit
Control PCR primer mix (for control reactions only)	2 µM of each control PCR primer 1 and 2	• Vial 6, PCR DIG Probe Synthesis Kit

Additionally required reagents

In addition to the reagents provided in the PCR DIG Probe Synthesis Kit and your template DNA, you will need the following reagents:

Additionally required reagent	Description
H_2O	Sterile, distilled water
Upstream Primer	1–10 µM upstream primer solution
Downstream Primer	1–10 µM downstream primer solution
Mineral oil	Mineral oil for overlaying amplification reactions (e.g., Sigma, Cat. No. M5904)

*This product is sold under licensing arrangements with Roche Molecular Systems and the Perkin-Elmer Corporation. For complete disclaimer, see inside back cover.

Procedure

❶ Add the following components to a sterile microcentrifuge tube. Place the tube on ice during pipetting. ▼

Reagents	Volume	Volume (Control Reaction)	Final Concentration
H_2O	variable	26.6 µl	–
PCR buffer without $MgCl_2$	5 µl	5 µl	1 x (10 mM Tris-HCl, 50 mM KCl)
$MgCl_2$ stock solution	2–10 µl	3 µl	1–5 mM
PCR DIG Probe Synthesis Mix	5 µl	5 µl	200 µM dNTP
Upstream Primer and Downstream Primer *or* Control PCR primer mix	variable –	– 5 µl	0.1–1 µM of each primer 0.2 µM of each primer
Taq DNA Polymerase	0.1–0.5 µl	0.4 µl	0.5–2.5 U/50 µl
Template DNA *or* Control template	variable* –	– 5 µl	variable 2 pg/µl
Total Volume	50 µl	50 µl	

*Consider the following template DNA amounts as guidelines when generating PCR probes for the detection of single-copy genes: human genomic DNA: 1–50 ng; plasmid DNA: 10–100 pg.

❷ Mix the reagents, and centrifuge briefly to collect the sample at the bottom of the tube.

❸ Overlay with 100 µl mineral oil to reduce evaporation of the mix, and amplify.
Cycling conditions depend on the respective template primers and the thermocycler. For general information about amplification conditions see reference 1. Cycling parameters for the control reaction are as follows:
Denature at 95°C for 7 min before the first cycle.
For 30 cycles:
Denature at 95°C for 45 sec
Anneal at 60°C for 1 min
Extend at 72°C for 2 min.
The control reaction generates an amplification product of 442 bp.

Notes

▶ *Optimization of reaction conditions*
PCR products can directly amplified and labeled from low amounts of genomic DNA (1 ng – 50 ng) and subsequently be used as hybridization probes. Optimal reaction conditions have to be adapted to each template/primer combination. In particular incubation times and temperatures, concentration of Mg^{2+} and enzyme but also concentration of template and primer should be optimized.

▶ *Analysis of PCR products*
After amplification analyse an aliquot of the reaction mixture (10 µl) by agarose gel electrophoresis. For use of the PCR product as a hybridization probe in genomic blots, a specific band should be visible after ethidium bromide staining following gel electrophoresis. Even minor amounts of by-products can influence the specificity of the hybridization when total genomic DNA is used as PCR template.

The control reaction generates an amplification product of 442 bp. Due to multiple incorporation of DIG-dUTP during the PCR process the molecular weight of the PCR product is increased significantly compared to the unlabeled product.

▶ *Removal/avoidance of unspecific by-products*
When using complex genomic templates for PCR DIG probe synthesis, the generation of even minor amounts of unspecific by-products influence significantly the specificity of hybridization to total genomic target DNA. This is due to the high sensitivity of the labeled probes generated during the PCR. Even in case no by-products are visible after ethidium bromide gel analysis of the

PCR product, we recommend to purify the labeled specific PCR product before using it as a hybridization probe on genomic blots. Separate the total PCR mixture using an agarose gel, cut the correct band from the gel and isolate the PCR fragment by established methods, like extraction with the Agarose Gel DNA Extraction Kit (Cat. No 1 696 505).

When using cloned plasmid templates for PCR DIG probe synthesis typically minor amounts of by-products do not influence the specificity of hybridization to genomic target DNA. In this case no purification of the PCR DIG probe is necessary. However, when major amounts of by-products are generated from cloned templates, we recommended to reduce the amount of template in the PCR reaction, thereby minimizing unspecific amplification.

▶ *Analysis of PCR products by direct detection*

When a blot is to be re-hybridized it is important that the signal from the previous hybridization can easily be removed. The PCR DIG probe synthesis mix (vial 2) therefore contains alkali-labile Digoxigenin-11-dUTP, which can be easily removed with an alkali stripping solution. The instability of the DIG probe in alkali-solutions do not allow that the PCR DIG Probe is transferred to membranes using alkali solutions. When you want to analyze the probe after electrophoresis and transfer to a membrane, you have to use neutral transfer solutions.

When the PCR product is not to be used as a probe but labeled with digoxigenin to allow sensitive detection of the PCR product itself, we recommend to use a dNTP mix with a lower Digoxigenin-11-dUTP:dTTP ratio. This is provided in the PCR DIG Labeling Mix (Cat. No. 1585550) where the ratio of Digoxigenin-11-dUTP (alkali-stable) to dTTP is 1:19.

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

Random Primed DNA Labeling

DNA can be labeled with Digoxigenin-11-dUTP using the random primed method. For optimal results, the template DNA should be linearized and purified by at least one phenol/chloroform extraction and ethanol precipitation prior to labeling. Templates of 100–10,000 bp label efficiently and produce probes with maximal sensitivity; therefore, templates >10 kb should be restriction-digested prior to labeling. For genomic Southern hybridizations, we recommend that you separate the insert from vector sequences before labeling.

Standard Random Primed DNA Labeling Reaction

The aim of the standard Random Primed DNA labeling reaction is to produce a sufficient amount of a digoxigenin-labeled probe in the shortest amount of time (1 hour); this 20 µl reaction will yield a minimum of 260 ng of digoxigenin-labeled probe from 1 µg of DNA template (see Table 3). In this standard reaction, one digoxigenin molecule is incorporated in every 20–25 nucleotides.

Products required

Most of the reagents required for random primed labeling are available separately, in the DIG DNA Labeling and Detection Kit (Cat. No. 1093 657), or in the DIG DNA Labeling Kit (Cat. No. 1175 033).

Name in procedure	Description	Available as
Hexanucleotide mixture (10 x)	62.5 A_{260} units/ml (1.56 mg/ml) random hexanucleotides, 500 mM Tris-HCl; 100 mM $MgCl_2$, 1 mM Dithioerythritol (DTE), 2 mg/ml BSA; pH 7.2	• Vial 5, DIG DNA Labeling and Detection Kit • Vial 5, DIG DNA Labeling Kit • Hexanucleotide Mix (Cat. No. 1277 081)
dNTP labeling mixture (10 x)	1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP; pH 6.5	• Vial 6, DIG DNA Labeling and Detection Kit • Vial 6, DIG DNA Labeling Kit • DIG DNA Labeling Mix (Cat. No. 1277 065)
Klenow enzyme, labeling grade	2 units/µl DNA Polymerase I (Klenow enzyme, large fragment) labeling grade, from *E. coli*	• Vial 7, DIG DNA Labeling and Detection Kit • Vial 7, DIG DNA Labeling Kit • Klenow enzyme (Cat. Nos. 1 008 404, 1 008 412)
Unlabeled Control DNA 2 (for control reaction only)	200 ng/ml pBR328 that has been linearized by *Eco* RI	• Vial 2, DIG DNA Labeling and Detection Kit • Vial 2, DIG DNA Labeling Kit

Additionally required solutions

In addition to the products above and your DNA template, you will need the following solutions.

Additionally required solution	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Template DNA	1 h	20 h
10 ng	15 ng	50 ng
30 ng	30 ng	120 ng
100 ng	60 ng	260 ng
300 ng	120 ng	500 ng
1000 ng	260 ng	780 ng
3000 ng	530 ng	890 ng

◀ **Table 2: Effect of template amount and labeling time on probe yield. The amount of synthesized DIG-labeled DNA increases with the amount of DNA template in the labeling reaction and the length of the incubation time at +37°C. Yields may vary from this example because of template purity, sequence, etc.**

Procedure

❶ Dilute 1 µg DNA template in H_2O to a total volume of 15 µl (10 ng–3 µg DNA template can be labeled with this procedure. Larger amounts can be labeled by scaling up of all components and volumes). For control reactions, mix 5 µl unlabeled control DNA 2 and 10 µl H_2O.

❷ Heat-denature the DNA template in a boiling water bath for 10 min, and quickly chill it on ice.
We have found that denaturation using a heating block is less effective and may result in lowered labeling efficiency.

❸ Add 2 µl Hexanucleotide mixture (10x) and 2 µl dNTP labeling mixture (10x) to the tube (on ice).

❹ Add 1 µl Klenow enzyme, labeling grade, for a final concentration of 100 U/ml, and mix.

❺ Incubate the reaction tube at +37°C for at least 60 min.
Longer incubations (up to 20 h) will increase the yield of DIG-labeled DNA (Table 2).

❻ Add 2 µl EDTA to the reaction tube.
This terminates the labeling reaction.

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-Labeled Nucleic Acids" protocol on page 33.

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 39.

Random Primed Labeling with DIG-High Prime

The DIG-High Prime (Cat No. 1585 606) offers a convenient alternative to random primed labeling with the DIG DNA Labeling and Detection Kit, DIG DNA Labeling Kit or individual labeling reagents. Sensitive DIG-labeled probes can be generated easily with this 5x concentrated labeling mixture of random hexamers, dNTP mix containing alkali-labile Digoxigenin-11-dUTP, labeling-grade Klenow enzyme, and an optimized reaction buffer. DIG-High Prime minimizes the hands-on time required to label DNA probes and eliminates most of the pipetting and mixing of reagents and buffers.

Products required

Name in procedure	Description	Available as
DIG-High Prime	DIG-High Prime (random hexamers, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile-digoxigenin-11-dUTP, 1 U/µl labeling-grade Klenow enzyme, 5x reaction buffer) in 50% (v/v) glycerol	• Cat. No. 1585 606

Additionally required solutions

In addition to DIG-High Prime and your DNA template, you will need the following solutions.

Additionally required solution	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Procedure

1. Dilute 1 µg DNA template (linear or supercoiled) in H_2O for a total volume of 16 µl. 10 ng–3 µg DNA template can be labeled with this procedure. When varying the amount of template DNA different amounts of DIG-labeled DNA are obtained (Table 3).
2. Heat-denature the DNA template in a boiling water bath for 10 min, and quickly chill it on ice.
   We have found that denaturation using a heating block is less effective and may result in lowered labeling efficiency.
3. Add 4 µl DIG-High Prime, mix, and centrifuge briefly.
4. Incubate the reaction tube at +37°C for at least 60 min.
   Longer incubations (up to 20 h) will increase the yield of DIG-labeled DNA (Table 3).
5. Add 2 µl EDTA to the reaction tube.
   This terminates the labeling reaction.

Yield of DIG-High Prime labeling reaction

Template DNA	1 h	20 h
10 ng	45 ng	600 ng
30 ng	130 ng	1050 ng
100 ng	270 ng	1500 ng
300 ng	450 ng	2000 ng
1000 ng	850 ng	2300 ng
3000 ng	1350 ng	2650 ng

Table 3:
Using the DIG-High Prime solution labeling reactions were performed with increasing amounts of different template DNAs for 1 h and 20 h.

Random Primed Labeling of DNA in Low Melting Point Agarose

DNA can also be labeled directly in low melting point agarose.

Procedure

1. Excise the DNA fragment to be labeled cleanly from a low melting point agarose gel and transfer it to a 1.5 ml microcentrifuge tube.
2. Add sterile redist. water to a ratio of 3 ml/g gel and heat the tube for 7 min at 100°C to melt the gel and denature the DNA.
3. After cooling to 37°C, the DNA/agarose mixture can be used directly for labeling in the standard procedures. The amount of hexanucleotides/dNTP mixture/Klenow enzyme or the amount of DIG-High Prime must be adjusted to the higher final volume.

Note: DNA labeled in agarose may not be subjected to ethanol precipitation, but must be purified by gel filtration.
We recommend to prolonge the incubation time to overnight in order to increase the yield of DIG labeled DNA.

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 39.

Nick Translation with Digoxigenin-11-dUTP

Nick translation is a well-established technique for labeling DNA probes. The reaction uses DNase I to create single-stranded nicks in double-stranded DNA. The 5'–3' exonuclease activity of *E. coli* DNA Polymerase I enters the nicks and removes stretches of single-stranded DNA; the degraded DNA is then replaced with labeled deoxyribonucleotides by the 5'–3' polymerase activity of the polymerase (1).

For routine blotting experiments, random-primed DNA labeling has perhaps overshadowed nick translation because of the higher specific activities obtained. However, nick translation is an especially useful labeling method for *in situ* hybridization experiments because it allows the lengths of the labeled DNA fragments to be controlled. Probe size is a critical parameter in *in situ* hybridization experiments because the probe has to be small enough to penetrate the tissue or cells.

Probe lengths of 200–500 bp are well suited for *in situ* hybridization experiments, and such probe lengths are readily obtained with the nick translation protocol below. The procedure was originally described by Rigby et al. (1) and tested for nucleotide analogues by Langer et al. (2).

Standard Labeling Reaction

Products required

Name in procedure	Description	Available as
DIG-Nick Translation Mix for *In situ* probes	5x conc. stabilized reaction buffer in 50% glycerol (v/v) and DNA Polymerase I, DNase I, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.17 mM dTTP and 0.08 mM DIG-11-dUTP	• Cat. No. 1745 816

Additionally required solutions

In addition, you will need the following solutions.

Additionally required solution	Description
EDTA	0.5 M EDTA, pH 8.0 (25°C)

Procedure

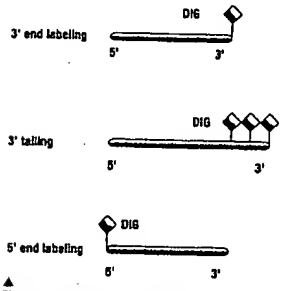
1. Place a 1.5 ml microcentrifuge tube on ice and add to the tube:
   16 µl sterile redistilled water containing 1 µg template DNA (not denatured, linear or supercoiled)
   4 µl DIG -Nick Translation Mix.
2. Mix ingredients and centrifuge the tube briefly.
3. Incubate at +15°C for 90 min.
4. Chill the reaction tube to 0°C.
5. Take a 3 µl aliquot from the tube and run the sample on an agarose minigel with a DNA molecular weight marker.
6. Depending on the average size of the probe, do one of the following:
   if the probe is between 200 and 500 nucleotides long, go to step 7.
   if the probe is longer than 500 nucleotides, incubate the reaction further at +15°C,. until the fragments are the proper size. *(Alternatively, the probe can be sonicated to obtain the proper size).*
7. Stop the reaction by adding 1 µl 0.5 M EDTA to the tube. Heat the tube to 65°C for 10 min.

Note: For nonradioactive nick translation with biotin incorporation we recommend the Biotin-Nick Translation Mix for in situ probes (Cat. No. 1745 824). For incorporation of fluorophore-labeled nucleotides, a Nick Translation Mix for in situ probes is available (Cat. No. 1745 808, fluorophore-labeled nucleotide must be purchased separately).

Chapter 3 • Oligonucleotide Labeling

A Comparison of Oligonucleotide-labeling Methods

Synthetic oligonucleotide probes are widely used in library-screening procedures, Southern and northern blots, dot blots, and *in situ* hybridization experiments. To provide researchers with maximum flexibility, Boehringer Mannheim has developed three methods for labeling oligonucleotides with digoxigenin (Figure 4).



▲ **Figure 4: Options for nonradioactive oligonucleotide labeling.**

This section briefly outlines the three oligonucleotide-labeling methods, each of which produces probes that are optimized for specific applications (see Table 4).

Oligonucleotide 3'-End Labeling (DIG Oligonucleotide 3'-End Labeling Kit)
The DIG Oligonucleotide 3'-End Labeling Kit (Cat. No. 1362372) is designed for the addition of Digoxigenin-11-ddUTP (DIG-ddUTP) to the 3' end of a synthetic oligonucleotide 14–100 nucleotides in length. The enzyme terminal transferase adds one digoxigenin residue per oligonucleotide because chain elongation cannot proceed past the dideoxy nucleotide. Each labeling reaction generates about 100 pmol of labeled probe (equal to 1 µg of a 30-mer). Probes labeled with this method retain their high degree of specificity and, despite the additional dideoxynucleotide, can still be treated under the same optimal hybridization and washing conditions (i.e., temperature and salt concentration). In addition, this method enables nonradioactive DIG labeling of conventionally synthesized oligonucleotides; therefore, the nonradioactive label DIG-ddUTP can be linked to the oligonucleotide without using any special reagents for oligonucleotide synthesis. These probes are particularly suited for experiments that require maximum probe specificity and moderate probe sensitivity (see Table 4).

Labeling method	Amount of oligo produced	Probe sensitivity	Probe specificity	Major characteristics	Application
3'-end labeling	100 pmol per reaction	++	+++	• Addition of a single DIG residue	Dot/slot blotting Colony/plaque hybridization Northern blots Southern blots
3' tailing	100 pmol per reaction	+++	++	• Addition of multiple DIG residues	*In situ* hybridization Northern blots Southern blots Dot/slot blots Colony/plaque hybridization
5'-end labeling with DIG-NHS ester	100 nmol per reaction	++	+++	• Oligo must be synthesized with aminolinker • Good for large-scale labeling • Chemical reaction	Sequencing Primer extension Northern blots Southern blots Colony/plaque hybridization Dot/slot blots

▲ **Table 4: Overview of Oligonucleotide-labeling methods.**

Oligonucleotide 3' Tailing (DIG Oligonucleotide Tailing Kit)

The DIG Oligonucleotide Tailing Kit (Cat. No. 1417231) is designed for the addition of a tail of residues ranging from 10–100 bases in length. In the 3' tailing reaction, terminal transferase adds a mixture of unlabeled nucleotides and Digoxigenin-11-dUTP, producing a tail containing multiple digoxigenin residues. The resulting probes are about ten times more sensitive than 3'-end labeled probes produced with the DIG Oligonucleotide 3'-End Labeling Kit.

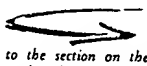
Although tailed oligonucleotide probes are more sensitive than 3'-end-labeled probes, they can also produce non-specific background due to the presence of the longer tail. For example, if the unlabeled nucleotide used in the tailing reaction is dATP, the probe may be inclined to anneal to T-rich regions in complex nucleic acid mixtures. Such non-specific signals can be minimized by choosing a different unlabeled nucleotide to utilize in the tailing reaction, by prehybridizing with a competing sequence (e.g., poly(A)), or by altering the stringency conditions.

5'-End Labeling Oligonucleotides (DIG Oligonucleotide 5'-End Labeling Set)

Oligonucleotides can be chemically tagged with digoxigenin at the 5' end in a two-step procedure with the DIG Oligonucleotide 5'-End Labeling Set (Cat. No. 1 480 863). In the first step, the oligonucleotide is synthesized with an aminolinker residue on its 5' end. After the synthetic oligonucleotide is purified, the second step involves the covalent linkage of digoxigenin-NHS ester to the free 5'-amino residue.

The resulting probes can be produced in large quantities (100 nmol per reaction), are specific, and have a sensitivity comparable to that of 3'-end-labeled probes (i.e., approximately 10 pg can be detected in a dot blot). The probes are suitable for applications such as library screening and dot blot hybridization experiments.

Another useful feature of 5'-end-labeled oligonucleotides is that the 3' end is free to act as a primer for DNA-synthesis reactions. Thus, extension reactions, such as PCR, can be conducted with labeled primers, allowing the nonradioactive tagging of the reaction products. Subsequently, the labeled extension products can be detected or be purified by affinity chromatography using anti-digoxigenin antibodies.



What to do next

At this time, proceed to the section on the appropriate oligonucleotide-labeling method to prepare your probe.

3'-End Labeling Oligonucleotides with Digoxigenin-11-ddUTP

Standard 3'-End Labeling Reaction

Products required

The DIG Oligonucleotide 3'-End Labeling Kit (Cat. No. 1 362 372) contains all of the components needed to make 3'-end-labeled oligonucleotide probes with digoxigenin. The kit also contains a DIG-ddUTP-labeled control oligonucleotide, which should be used in a direct detection assay to estimate the yield of DIG-labeled oligonucleotide (see page 33), as well as an unlabeled control oligonucleotide for labeling, and a control DNA for hybridization. The DIG-ddUTP-labeled control oligonucleotide and kit components required for labeling are also available as separate products.

*Potassium cacodylate is toxic. Wear gloves when handling. Discard as regulated for toxic waste.

Name in procedure	Description	Available as
5 x reaction buffer	1 M potassium cacodylate*, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6 (+25°C)	• Vial 1, DIG Oligonucleotide 3'-End Labeling Kit • Supplied with terminal transferase
$CoCl_2$ solution	25 mM cobalt chloride ($CoCl_2$)	• Vial 2, DIG Oligonucleotide 3'-End Labeling Kit • Supplied with terminal transferase
DIG-ddUTP	1 mM Digoxigenin-11-ddUTP (2',3'-dideoxyuridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in redistilled water	• Vial 2, DIG Oligonucleotide 3'-End Labeling Kit • DIG-ddUTP (Cat. No. 1 363 905)
Terminal Transferase	50 units/µl terminal transferase, in 200 mM potassium cacodylate*, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% (v/v) glycerol; pH 6.5 (+25°C)	• Vial 4, DIG Oligonucleotide 3'-End Labeling Kit • Terminal Transferase [Cat. No. 220 582 (sold at 25 U/µl; contains 5 x reaction buffer and cobalt chloride)]
Unlabeled Control Oligonucleotide (for control reactions only)	30-mer, 5'-pTTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids, in redistilled water	• Vial 5, DIG Oligonucleotide 3'-End Labeling Kit

Additionally required solution	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Additionally required solutions

In addition to the DIG Oligonucleotide 3'-End Labeling Kit and your oligonucleotide, you will need the following solutions.
◀

Procedure

Purify (by HPLC or gel electrophoresis) the oligonucleotide to be labeled after synthesis. Most suppliers will do this for the customer.

❶ Add reagents to a sterile microfuge tube (on ice) in the following order:
◀

Reagents	Volume	Volume (Control Reaction)	Final Concentration
5 x Reaction buffer	4 µl	4 µl	1 x
$CoCl_2$ solution	4 µl	4 µl	5 mM
Oligonucleotide	variable (100 pmol)	–	5 pmol/µl
or Unlabeled Control Oligonucleotide	–	5 µl (100 pmol)	5 pmol/µl
DIG-ddUTP	1 µl	1 µl	0.05 mM
Terminal Transferase	1 µl	1 µl	2.5 units/µl
H_2O	to 20 µl	5 µl	–
Total Volume	20 µl	20 µl	

❷ Incubate the reaction at +37°C for 15 min. Place on ice.

❸ Add 1 µl EDTA to the reaction tube. This terminates the labeling reaction.

Notes on subsequent hybridization

▶ The labeled probe may be diluted in hybridization buffer without ethanol precipitation. The presence of unincorporated DIG-ddUTP will not cause a background problem if SSC buffer is used for hybridization and wash steps. However, if TMAC is used in the wash and hybridization buffer, or if the probe is to be used in *in situ* hybridization, ethanol precipitate the labeled probe (see page 39).

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 39.

3' Tailing Oligonucleotides with Digoxigenin-11-dUTP/dATP

Standard Tailing Reaction

Products required

The DIG Oligonucleotide Tailing Kit (Cat. No. 1417231) contains all of the components needed to make tailed probes containing DIG-dUTP/dATP residues. Nucleotides other than the dATP may be used to tail oligonucleotides; see page 27 for the modified procedure. The kit also contains a DIG-dUTP/dATP-tailed control oligonucleotide, which should be used in a direct detection assay to estimate the yield of DIG-labeled oligonucleotide (see page 33). In addition the kit contains an unlabeled control oligonucleotide for labeling and a control DNA for hybridization. The kit components required for tailing are also available as separate items.

Name in procedure	Description	Available as
5 x Reaction buffer	1 M potassium cacodylate*, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6 (+25° C)	• Vial 1, DIG Oligonucleotide Tailing Kit • Supplied with terminal transferase
$CoCl_2$ solution	25 mM cobalt chloride ($CoCl_2$)	• Vial 2, DIG Oligonucleotide Tailing Kit • Supplied with terminal transferase
DIG-dUTP	1 mM Digoxigenin-11-dUTP (2'-dideoxyuridine-5'-triphosphate, coupled to digoxigenin via an 11-atom spacer arm) in redistilled water	• Vial 3, DIG Oligonucleotide Tailing Kit • DIG-dUTP, alkali-labile (Cat. Nos. 1573152, 1573179) • DIG-dUTP, alkali-stable (Cat. Nos. 1093088, 1558706, 1570013)
dATP	10 mM dATP solution; in Tris buffer, pH 7.5	• Vial 4, DIG Oligonucleotide Tailing Kit • dATP [Cat. No. 1051440 (sold as a 100 mM solution; must be diluted before use)]
Terminal Transferase	50 units/µl terminal transferase, in 200 mM potassium cacodylate*, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% (v/v) glycerol; pH 6.5 (+25° C)	• Vial 5, DIG Oligonucleotide Tailing Kit • Terminal Transferase [Cat. No. 220582 (sold as 0.25 U/µl; contains 5x reaction buffer and cobalt chloride)]
Unlabeled Control Oligonucleotide (for control reactions only)	30-mer, 5'-pTTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z'-region in pUC and M13 plasmids, in redistilled water	• Vial 6, DIG Oligonucleotide Tailing Kit

*Potassium cacodylate is toxic. Wear gloves when handling. Discard as regulated for toxic waste.

Additionally required solutions

In addition to the DIG Oligonucleotide Tailing Kit and your oligonucleotide, you will need the following solutions.

Additionally required solution	Description
H_2O	Sterile, distilled water
EDTA	200 mM EDTA, pH 8.0

Procedure

The oligonucleotide to be labeled should be purified by HPLC or gel electrophoresis after synthesis. Most suppliers will do this for the customer.

❶ Add reagents to a sterile microfuge tube (on ice) in the following order: ▼

Reagents	Volume	Volume (Control Reaction)	Final Concentration
5x Reaction buffer	4 µl	4 µl	1 x
$CoCl_2$ solution	4 µl	4 µl	5 mM
DIG-dUTP	1 µl	1 µl	0.05 mM
Oligonucleotide	variable (100 pmol)	–	5 pmol/µl
or Unlabeled Control Oligonucleotide	–	5 µl (100 pmol)	5 pmol/µl
dATP	1 µl	1 µl	0.5 mM
Terminal Transferase	1 µl	1 µl	2.5 units/µl
H_2O	to 20 µl	4 µl	–
Total Volume	20 µl	20 µl	

When upscaling the labeling reaction all components have to be increased proportionally. Increasing only the oligonucleotide concentration results in inefficient labeling.

❷ Incubate the reaction at +37°C for 15 min and then place on ice.

❸ Add 1 µl EDTA to the reaction tube. This terminates the labeling reaction.

Notes on subsequent hybridization

▶ We have occasionally experienced background problems when probes labeled in this manner are used in hybridization buffers containing TMAC. Hybridization buffers containing SSC are preferable when "long-tailed" probes are used. If it is necessary to use TMAC, we recommend the use of an oligonucleotide probe labeled with DIG-ddUTP as well as ethanol precipitation before use.

▶ Use of tailed probes in hybridization experiments can sometimes cause nonspecific hybridization of the tail to complementary sequences in the target DNA. To prevent this, add 0.1 mg/ml poly(A) (vial 11 or Cat. No. 108 626) and/or 5 µg/ml poly(dA) (Cat. No. 223 581) to the prehybridization and hybridization buffer; this will block the target sequences.

▶ Both the optimal concentration of labeled probe in the hybridization buffer and the time required for hybridization depend on the amount of DNA or RNA that will be detected on a filter. Usually, the probe is diluted to 1–10 pmol/ml, and the hybridization is carried out for 1–6 hours in at least 3.5 ml of hybridization buffer per 100 cm^2 of membrane.

▶ Heat-denature the oligonucleotide prior to hybridization if secondary structure can be expected from the oligonucleotide sequence.

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 39.

Oligonucleotide Tailing with Nucleotides Other than dATP

Oligonucleotides can also be tailed with DIG-dUTP and dGTP, or dCTP and dTTP, or a mixture of all four unlabeled deoxynucleoside triphosphates. See Table 5 for tail lengths and incorporation rates for other nucleotides. ▶

DIG-dUTP/dNTP labeling mixture, 1:10	dATP	dCTP	dGTP	dTTP	dNTP
Average tail length	50	25	15	10	5
Range of tail length	10–100	10–40	10–25	1–20	1–10
DIG-dUTP/tail	5	2.5	1.5	1	0.5

▲ **Table 5: Tail-lengths and incorporation rates for other nucleotides**

Products required

In addition to the DIG Oligonucleotide Tailing Kit (Cat. No. 1 417 231), the following products can be purchased separately when tailing oligonucleotides with other nucleotides. ▼

Available reagent	Description	Available as
dCTP	100 mM dCTP, lithium salt	• dCTP (Cat. No. 1 051 458)
dGTP	100 mM dGTP, lithium salt	• dGTP (Cat. No. 1 051 466)
dTTP	100 mM dTTP, lithium salt	• dTTP (Cat. No. 1 051 482)
Deoxynucleotide Triphosphate Set	dATP, dCTP, dGTP, dTTP; 100 mM each; pH 7.0	• Deoxynucleotide Triphosphate Set (Cat. No. 1 277 049)

Procedure

❶ Mix 9 volumes of DIG-dUTP with 1 volume of the appropriate deoxynucleotide triphosphate solution (to be chosen from the "Available reagent" list (above)). This DIG-dUTP/dNTP tailing mixture will be added to the oligonucleotide tailing reaction in place of the DIG-dUTP and dATP.

❷ Add reagents to a sterile microfuge tube (on ice) in the following order: ▶

Reagents	Volume	Volume (Control Reaction)	Final Concentration
5 x Reaction buffer	4 µl	4 µl	1 x
$CoCl_2$ solution	4 µl	4 µl	5 mM
DIG-dUTP/dNTP tailing mixture	1 µl	1 µl	0.5/0.5 mM
Oligonucleotide	variable (100 pmol)	–	5 pmol/µl
or Unlabeled Control Oligonucleotide	–	5 µl (100 pmol)	5 pmol/µl
Terminal Transferase	1 µl	1 µl	2.5 units/µl
H_2O	to 20 µl	5 µl	–
Total Volume	20 µl	20 µl	

❸ Incubate the reaction at + 37°C for 15 min. Place on ice.

❹ Add 1 µl EDTA to the reaction tube. This terminates the labeling reaction.

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 39.

5'-End Labeling Oligonucleotides with Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (DIG-NHS ester)

Standard 5'-End Labeling Reaction

With the DIG Oligonucleotide 5'-End Labeling Set (Cat. No. 1480863), oligonucleotides can be labeled with digoxigenin at the 5'-end after synthesis that includes the addition of a phosphoramidite. First, oligonucleotides are reacted with the phosphoramidite in a final synthesis step according to the solid-phase phosphoramidite method, creating a 5'-terminal amino function. Treatment with ammonia releases the oligonucleotide from the support and cleaves the protecting groups. In the subsequent step, digoxigenin is introduced at the 5'-end.

Name in procedure	Description	Available as
Aminolinker*	[N-Trifluoroacetamido-(3-oxa)-pentyl-N,N-diisopropyl-methyl]-phosphoramidite	• Vial 1, DIG Oligonucleotide 5'-End Labeling Set • Aminolinker (Cat. No. 1685643)
DIG-NHS ester**	Digoxigenin-3-O-methylcarbonyl-ε-amino-caproic acid-N-hydroxysuccinimide ester	• Vial 2, DIG Oligonucleotide 5'-End Labeling Set • DIG-NHS ester (Cat. No. 1333054)

* The aminolinker reacts violently with water, and it is irritating to eyes, respiratory system, and skin.
** DIG-NHS ester is very toxic by inhalation, in contact with skin, or swallowed. Do not breath dust.

Additionally required solutions

In addition to the DIG Oligonucleotide 5'-End Labeling Set, you will need the following solutions.

Additionally required solution	Description
Acetonitrile	Anhydrous acetonitrile
Aqueous ammonia	25% aqueous ammonium
H_2O	Sterile, redist. water
Sodium acetate	3 M sodium acetate, pH 8.5
Ethanol	Absolute ethanol, chilled at –20°C, when 70% ethanol is indicated, dilute ethanol with redistilled water
Sodium borate	100 mM sodium borate; pH 8.5
Elution buffer A	100 mM triethylammonium acetate; pH 6.8
Elution buffer B	100 mM triethylammonium acetate:acetonitrile (1:1); pH 6.8

Introduction of the 5' amino function

The Aminolinker used corresponds to the phosphoramidites used in oligonucleotide synthesis protocol.

The bottle with the Aminolinker fits directly into the appropriate position of an automatic DNA synthesizer from Applied Biosystems, Pharmacia, or Eppendorf.

For use in the synthesizers from Milligen/Biosearch, the bottle content has to be dissolved in the appropriate amount of anhydrous acetonitrile (see below) by injection of the solvent into the sealed bottle with a disposable syringe and subsequent transfer to the reservoir at the synthesizer.

❶ Dissolve 100 mg Aminolinker in anhydrous acetonitrile for the synthesizers from Applied Biosystems, Pharmacia, Eppendorf in 2.7 ml (100 mM); Milligen/Biosearch in 4.3 ml (70 mM).
Note: The solution of the phosphoramidite is stable for approx. 2 weeks at ambient temperature and exclusion of moisture.

❷ Start oligonucleotide synthesis according to standard protocol. Set the synthesizer on "trityl on".

❸ Deprotect the oligonucleotide according to standard oligonucleotide synthesis (by treatment with 25% aqueous ammonia).

❹ Remove ammonia by evaporation or lyophilization.

Ethanol precipitation of the oligonucleotide

1. Dissolve the oligomer (approx. 100 nmol) in a mixture of 300 µl H_2O and 30 µl 3 M sodium acetate, pH 8.5, and transfer into a microfuge tube.
2. Add 0.9 ml ice-cold ethanol. Mix well.
3. Incubate at –20°C for 2–3 h.
4. Centrifuge for 15 min at 10,000 x g. Decant the supernatant.
5. Wash the pellet with 100 µl of ice-cold ethanol, centrifuge for 5 min at 10,000 x g, and decant the supernatant.

Labeling reaction (example of a 20-mer)

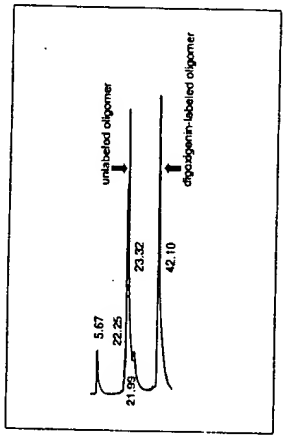
1. Dissolve the oligonucleotide pellet (approx. 20 A_{260} units, corresponding to approx. 100 nmol) in 200 µl sodium borate.
2. Dissolve the content of a vial (1 mg) of the DIG-NHS ester in 600 µl ethanol, and add 200 µl of this solution to the oligonucleotide solution.
   Note: The solution of the DIG-NHS ester in ethanol is only stable for a short time. Therefore, we recommend that you use the ester solution for multiple parallel labelings (e.g., in this case, for two additional reactions).
   In general, 1 mg (1.5 µmol) of the DIG-NHS ester is sufficient for labeling 300 nmol of 5'-amino-substituted oligonucleotide. Starting with 20 A_{260} units of oligomer, 1 mg DIG-NHS ester is sufficient for
   2 labeling reactions of a 15-mer
   3 labeling reactions of a 20-mer
   4 labeling reactions of a 25-mer
3. Place vials on a shaker platform overnight at room temperature.

Purification of the labeled oligonucleotide

Separation of the labeled oligonucleotide from the unlabeled compound may be achieved by reversed phase HPLC*.

1. Concentrate the mixture of the labeling reaction under vacuum.
2. Dissolve the remainder in 1 ml of H_2O.
3. Pass this mixture through a 0.45 µm filter.
4. Apply onto a HPLC column, RP-18/ 5 µm.
   Gradient: In 30 min from 100% elution buffer A to 80% elution buffer B.
   The digoxigenin-labeled oligonucleotide is eluted with a higher retention value compared to the unlabeled compound. A typical elution profile is shown in Figure 5. An average yield of 50% digoxigenin-labeled oligomers is obtained.
5. Concentrate the appropriate fraction under vacuum. Desalt as usual (e.g., gel filtration, dialysis in SPECTRAPOR® 1000).

* Alternatively, the separation from unlabeled oligonucleotides can be achieved by standard polyacrylamide gel electrophoresis. As an additional alternative, the labeled oligonucleotide may be purified on PR-C-18 cartridges (e.g., Poly-Pak RP1 from MWG Biotech, Roth; OPC from ABI).



◀ **Figure 5: Elution profile.** Oligonucleotides were purified after labeling with DIG-NHS ester by reversed phase HPLC with an Inertsil™ column. Columns from other manufacturers give similar elution profiles, though the distance between the peaks can vary.
A: unlabeled oligonucleotide
B: DIG-labeled oligonucleotide

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

Chapter 4 • RNA Labeling

Labeling RNA with the DIG RNA Labeling Kit

The DIG RNA Labeling Kit generates DIG-labeled, single-stranded RNA probes of defined length by transcription. RNA probes are labeled with digoxigenin, using SP6, T7, or T3 RNA Polymerases. DNA is subcloned into a multiple cloning site adjacent to the RNA polymerase promoter site in the pSPT18 or pSPT19 transcription vectors provided in the kit. A restriction enzyme (not provided in the kit) linearizes the DNA template, allowing the creation of "run off" transcripts to uniform length. One Digoxigenin-11-UTP residue is incorporated every 20–25 nucleotides.

A large amount of DIG-labeled RNA can be generated by this method because the nucleotide concentration does not become limiting in the standard transcription assay. Under standard conditions, approximately 2–10 µg of full-length DIG-labeled RNA is transcribed from 1 µg of template DNA.

The RNA probes produced with this method are desirable because they:

- have a defined unit length
- exhibit single-strand target specificity
- do not re-anneal like double-stranded DNA probes.

RNA labeled with digoxigenin is particularly useful for Northern blots. In addition, DIG-labeled RNA probes can be used for Southern blots, plaque or colony screening, and *in situ* hybridization. Also, because the linkage between DIG and UTP is resistant to alkali, DIG-labeled RNA can be fragmented by alkaline treatment. When creating RNA probes for *in situ* hybridization, a limited reduction in size of the DIG-labeled RNA probe can be advantageous (see the section entitled "Regulation of RNA Probe Length by Alkaline Hydrolysis" on page 32 for details).

Standard Labeling Reaction

Products required

The most convenient approach to making RNA probes is to use the DIG RNA Labeling Kit (SP6/T7) (Cat. No. 1 175 025) because it contains most of the components needed to make RNA probes. The kit also contains a number of control RNAs and DNAs, which can be used in hybridizations, direct detections, or to check the efficiency of the labeling reaction (see page 33). Some of the kit components are also available as separate products, but the buffers and some of the controls are not.

Name in procedure	Description	Available as
NTP labeling mixture (10x)	10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP; in Tris-HCl, pH 7.5 (+20°C)	• Vial 7, DIG RNA Labeling Kit (SP6/T7) • DIG RNA Labeling Mix (Cat. No. 1 277 073)
10x Transcription buffer	400 mM Tris-HCl, pH 8.0; 60 mM $MgCl_2$, 100 mM dithioerythritol (DTE), 20 mM spermidine, 100 mM NaCl, 1 unit/ml RNase inhibitor	• Vial 8, DIG RNA Labeling Kit (SP6/T7)
DNase I, RNase-free	10 units/µl DNase I, RNase-free	• Vial 9, DIG RNA Labeling Kit (SP6/T7) • DNase I, RNase-free (Cat. No. 776 785)
RNase inhibitor	20 units/µl RNase inhibitor	• Vial 10, DIG RNA Labeling Kit (SP6/T7) • RNase inhibitor (Cat. Nos. 799 017, 799 025)
One of the following		
SP6 RNA Polymerase	20 units/µl SP6 RNA Polymerase	• Vial 11, DIG RNA Labeling Kit (SP6/T7) • SP6 RNA Polymerase (Cat. Nos. 810 274, 1 487 671)
T7 RNA Polymerase	20 units/µl T7 RNA Polymerase	• Vial 12, DIG RNA Labeling Kit (SP6/T7) • T7 RNA Polymerase (Cat. Nos. 881 767, 881 775)
T3 RNA Polymerase	20 units/µl T3 RNA Polymerase	• T3 RNA Polymerase (Cat. Nos. 1 031 163, 1 031 171)
One of the following (for control reactions only):		
Control DNA 1, pSPT-18 Neo or	0.25 mg/ml pSPT-18 Neo DNA, cleaved with *Pvu* II	• Vial 3, DIG RNA Labeling Kit (SP6/T7)
Control DNA 2, pSPT-19 Neo	0.25 mg/ml pSPT-19 Neo DNA, cleaved with *Pvu* II	• Vial 4, DIG RNA Labeling Kit (SP6/T7)

Additionally required solutions

In addition to the DIG RNA Labeling Kit and your purified DNA template, you will need the following solutions.

Additionally required solution	Description
DMPC-treated H_2O	Sterile, distilled water treated with 0.1% dimethylpyrocarbonate (see page 84)
EDTA	200 mM EDTA, pH 8.0

Procedure

Before beginning the transcription reaction, the DNA template must be linearized at a restriction enzyme site downstream of the cloned insert. To avoid transcription of undesirable sequences, use a restriction enzyme that leaves 5' overhangs or blunt ends. After the restriction digest, purify the DNA by phenol/chloroform extraction, followed by ethanol precipitation. Alternatively, we have found Elutip™-r columns (Schleicher & Schuell; Cat. No. NA 020/2) to be an easy and effective method of purifying DNA template without contaminating the sample with RNase.

❶ Add reagents to a sterile, RNase-free microfuge tube (on ice) in the following order: ▼

Reagents	Volume	Volume (Control Reaction)	Final Concentration
Purified DNA template *or*	variable (1 µg)	–	0.05 µg/µl
Control DNA 1 or 2	–	4 µl	0.05 µg/µl
NTP labeling mixture (10 x)	2 µl	2 µl	1 x
10 x Transcription buffer*	2 µl	2 µl	1 x
DMPC-treated H_2O	to 18 µl	10 µl	–
RNA Polymerase (SP6, T7, or T3)	2 µl	2 µl	2 units/µl
Total Volume	20 µl	20 µl	

*Optional: Add an additional 1 µl of RNase Inhibitor (vial 10).

❷ Mix gently and centrifuge briefly. Incubate for at least 2 hours at + 37°C.

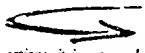
❸ If desired, add 2 µl DNase I, RNase-free (vial 9 or Cat. No. 776785), and incubate for 15 minutes at + 37°C to remove the DNA template.
Because the amount of DIG-labeled RNA transcript greatly exceeds the amount of DNA template, removal of the DNA template is usually unnecessary.

❹ With or without prior DNase treatment, add 2 µl EDTA solution to the reaction tube. This terminates the transcription reaction.

The reaction may be scaled up to increase the yield of RNA. This is achieved by keeping the amount of template DNA constant while increasing the amount of the other components in the labeling reaction. For example, in a 5 x scaled-up reaction with 1 µg of linear control DNA 1 (pSPT18-Neo DNA) as template, more than 40 µg of RNA can be synthesized after a two-hour incubation at + 37°C.

The amount of newly synthesized DIG-labeled RNA depends on the amount, size (site of linearization), and purity of the template DNA. When 1 µg of template DNA that has been linearized to give run off transcripts of 760 bases is labeled in the standard reaction, approximately 37% of the nucleotides are incorporated into about 10 µg of transcribed DIG-labeled RNA.

The RNA transcripts can be analyzed for size by agarose gel electrophoresis (e.g., formaldehyde gels) and ethidium bromide staining. Labeling efficiency can be most accurately checked by direct detection of the labeled RNA probe with Anti-Digoxigenin-alkaline phosphatase.



What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

For filter hybridizations, it is usually not necessary to clean up the probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for in situ hybridization, see page 39.

Regulation of RNA Probe Length by Alkaline Hydrolysis

Some applications require shorter RNA probes than other techniques. When performing *in situ* hybridizations, for example, probes must be short enough to allow diffusion into and out of the tissue. Alkaline hydrolysis allows you to regulate the size of RNA probes.

Additionally required solutions

Additionally required solution	Description
DMPC-treated H_2O	Sterile, distilled water treated with 0.1% dimethylpyrocarbonate (see page 84)
Carbonate buffer	60 mM Na_2CO_3; 40 mM $NaHCO_3$; pH 10.2
Hydrolysis-neutralization buffer	200 mM sodium acetate; 1% (v/v) acetic acid; pH 6.0
Ethanol	Absolute ethanol, chilled at –20°C; when 70% ethanol is indicated, dilute the ethanol with DMPC-treated water.

Procedure

The following procedure is a modification of the protocol regulating the size of RNA probes by alkaline hydrolysis described by Cox, et al. (1984, *Develop. Biol.* 101, 485–502). This protocol was adapted for use with DIG-UTP-labeled RNA probes.

1. Hydrolyze 1 µg RNA by adding an equal volume of DMPC-treated H_2O and two volumes of carbonate buffer. Incubate for 10–60 min at +60°C. The optimal incubation time must be determined empirically. We have found that hydrolysis starts as early as 30 s after the addition of the carbonate buffer.
2. Add an equal volume of hydrolysis-neutralization buffer to stop the hydrolysis.
3. Add 3 volumes of chilled ethanol to precipitate the RNA. Mix well and incubate at –70°C for 30 min.
4. Centrifuge at 13,000 x g for 15 min at +4°C in a microcentrifuge.
5. Decant the ethanol, and wash the pellet with 100 µl of cold 70% ethanol. Centrifuge at 13,000 x g for 5 min at +4°C in the microcentrifuge, then remove the 70% ethanol.
6. Dry the pellet and resuspend in 100 µl DMPC-treated H_2O. If not used immediately, store the probe at –70°C.
7. Check the resulting probe length by electrophoresis of 10 µl hydrolyzed RNA on a 1% ethidium bromide-stained agarose gel.

Chapter 5 • Estimating the Yield of DIG-Labeled Nucleic Acids

An accurate quantification of DIG-labeled DNA obtained in the labeling reaction is most important for optimal and reproducible results in various membrane hybridization techniques. Too high of a probe concentration in the hybridization mix usually causes background, while too low of a concentration leads to weak signals.

There are two ways to estimate the yield of DIG-labeling. The first option is a 30 min procedure in which dilutions of the labeling reaction are spotted on DIG Quantification Teststrips. After detection the intensities are compared to a simultaneous detected DIG Control Teststrip, that has defined amounts of DIG-labeled DNA already spotted on it.

In the second procedure dilution series of the labeling reaction and dilutions of an appropriate standard are both spotted on nylon membranes. The membrane is then processed in a short detection procedure.

Estimating the yield with DIG Quantification and DIG Control Teststrips

Quantification using teststrips consists of two basic steps. First, a series of dilutions of DIG-labeled DNA is applied to the squares on the DIG Quantification Teststrips. DIG Control Teststrips are already loaded with 5 defined dilutions of a control DNA and are used as standards.

The teststrips are then subjected to immunological detection with Anti-Digoxigenin-AP and the color substrates NBT/BCIP. After approx. 30 min the test procedure is completed and the DIG-labeling efficiency can be determined by comparing the signal intensities of the spots on the quantification teststrip with the control teststrip.

Products required

Name in procedure	Description	Available as
DIG Quantification Teststrips	Teststrips of 0.6 x 8 cm, coated with positively charged nylon membrane, unloaded	• DIG Quantification Teststrips (Cat. No. 1669958)
DIG Control Teststrips	Teststrips of 0.6 x 8 cm, coated with positively charged nylon membrane, loaded with DIG labeled control DNA in the quantities 300, 10, 30, 10 and 3 pg	• DIG Control Teststrips (Cat. No. 1669966)
DNA Dilution buffer	10 mM Tris-HCl, pH 8.0 (20°C), 50 µg/ml DNA from herring sperm	• Vial 3, DIG DNA Labeling Kit • Vial 3, DIG DNA Labeling and Detection Kit • Vial 2, DIG Nucleic Acid Detection Kit • Vial 9, DIG Oligonucleotide 3'-End Labeling Kit • Vial 10, DIG Oligonucleotide Tailing Kit
RNA Dilution buffer	DMPC-treated H_2O, 20 x SSC and formaldehyde, mixed in a volume ratio of 5 ÷ 3 ÷ 2	
Blocking Reagent	Blocking reagent for nucleic acid hybridization; white powder	• Vial 11, DIG DNA Labeling and Detection Kit • Vial 6, DIG Nucleic Acid Detection Kit • Blocking Reagent (Cat. No. 1096176)
Anti-Digoxigenin-AP	Anti-digoxigenin (Fab) conjugated to alkaline phosphatase	• Vial 6, DIG DNA Labeling and Detection Kit • Vial 3, DIG Nucleic Acid Detection Kit • Anti-Dipoxigenin-AP, Fab fragments (Cat. No. 1093274)
NBT solution	75 mg/ml nitroblue tetrazolium salt in dimethylformamide	• Vial 9, DIG DNA Labeling and Detection Kit • Vial 4, DIG Nucleic Acid Detection Kit • NBT (Cat. No. 1383213 (dilute from 100 mg/ml))
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in dimethylformamide	• Vial 10, DIG DNA Labeling and Detection Kit • Vial 5, DIG Nucleic Acid Detection Kit • BCIP (Cat. No. 1383221)

Additionally required solutions

Except TE buffer, all of the following required solutions are available in a ready-to-use form in the DIG Wash and Block Buffer Set (Cat. No 1585762). Bottle numbers for this set are indicated in parentheses. Alternatively they can be prepared from separate reagents according to procedures described in Appendix B.

Additionally required solutions	Description
Washing buffer (Bottle 1; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 0.3% (v/v) Tween® 20
Maleic acid buffer (Bottle 2; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking solution (Bottle 3; dilute 1:10 with 1 x maleic acid buffer)	1% (w/v) Blocking reagent for nucleic acid hybridization, dissolved in Maleic acid buffer. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4°C, but must then be brought to room temperature before use
Detection buffer (Bottle 4; dilute 1:10 with H_2O)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (+20°C)
TE buffer	10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0 (+20°C)

Procedure

Preparation of a dilution series

❶ Dilute your labeling reaction to an approximate concentration of 1 µg/ml.
The approximate concentration of your DIG-labeled DNA can be estimated according to the standard yields of the respective labeling kit or reagent used, that are given in the pack insert. (Example: A DIG-High Prime reaction yields approx. 40 µg/ml of DIG-labeled DNA after 1 h incubation, starting from 1 µg template. Dilute 1 µl of your labeling reaction with 39 µl DNA dilution buffer to obtain a final concentration of 1 µg/ml)

❷ Dilute the 1 µg/ml predilution from step 1 according to the following scheme: ▼

Dilution steps	Dilution in DNA dilution buffer	Final Concentration	Name of dilution
1. 1:3.3	10 µl + 23 µl buffer	300 pg/µl	A
2. 1:10	5 µl + 45 µl buffer	100 pg/µl	B
3. A diluted 1:10	5 µl A + 45 µl buffer	30 pg/µl	C
4. B diluted 1:10	5 µl B + 45 µl buffer	10 pg/µl	D
5. C diluted 1:10	5 µl C + 45 µl buffer	3 pg/µl	E

❸ Apply a 1 µl spot of dilutions A–E onto the marked squares of a DIG Quantification Teststrip.
If you want to mark the teststrip use the polyester carrier area. Avoid writing on the membrane itself. Touch only with gloves.

❹ Airdry for approx. 2 min.

Preparation for the detection procedure

The small format of the teststrips allows that only very low volume of test solutions must be used.

❺ Prepare an antibody solution by diluting 1 µl of Anti-Digoxigenin-alkaline phosphatase in 2 ml blocking solution.

❻ Prepare color-substrate by adding 9 µl NBT solution and 7 µl BCIP solution to 2 ml of detection buffer

❼ For each detection series prepare 5 microcuvettes or 2.5 ml reaction vials and label them from 1 to 5.
To vial 1: add 2 ml of blocking solution
To vial 2: add 2 ml of antibody solution (prepared under 5.)
To vial 3: add 2 ml of washing buffer
To vial 4: add 2 ml of detection buffer
To vial 5: add 2 ml of color-substrate solution (prepared under 6.)

Detection

Note: This short detection protocol is exclusively suited for the quantification of DIG-labeled nucleic acids. It cannot replace the standard detection protocol given in this manual or in the pack inserts of the DIG detection kits or substrates. The limits of detection using the short protocol are a factor 10–30 below the sensitivity achieved with the standard detection protocols.

❽ Dip the prepared teststrips (one Quantification Teststrip and one Control Teststrip should be developed back to back in one vial) in the prepared solution in the following sequence and for the given incubation times. Between steps, let excessive fluid drip onto a paper tissue.

vial 1	blocking	2 min
vial 2	antibody binding	3 min
vial 1	blocking	1 min
vial 3	washing	1 min
vial 4	equilibration	1 min
vial 5	color reaction (in the dark)	5–30 min

❹ Stop the color reaction after a maximum of 30 min by briefly rinsing the teststrips in water. Air dry on Whatman 3 MM paper, protected from light. Extended color reaction time leads to increased background.

Evaluation of results

The first spots should be visible after 5–10 min of the color reaction; the 30 pg spot should be visible by then. After 30 min incubation the 3 pg spot should be visible on both the Quantification and the Control Teststrip.
You can now determine the quantity of DIG labeled DNA or RNA in the squares of the Quantification Teststrip by comparing the color intensity with the Control Teststrip. Calculate the quantity of DIG-labeled DNA in your labeling reaction by taking the dilution steps into account.

Estimating the yield in a spot test with a DIG-labeled control

The estimation of yield can also be performed in a side by side comparison of the DIG-labeled sample nucleic acid with a DIG-labeled control, that is provided in the labeling kits. Dilution series of both are prepared and spotted on a piece of membrane. Subsequently, the membrane is colorimetrically detected. Direct comparison of the intensities of sample and control allows the estimation of labeling yield.

Products required

DIG-labeled controls for estimating the yield of DNA, RNA and end-labeled oligonucleotides are available as separate reagents or in the respective labeling kits. The DIG-dUTP/dATP-tailed Oligonucleotide Control is only available in the DIG Oligonucleotide Tailing Kit.

DIG-Labeled Control	Description	Available as
Labeled Control DNA	Digoxigenin-labeled pBR328 DNA that has been random primed labeled according to the standard labeling procedure; the total DNA concentration in the vial is 25 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA	• Vial 4, DIG DNA Labeling and Detection Kit • Vial 4, DIG DNA Labeling Kit • Vial 1, DIG Nucleic Acid Detection Kit • DIG-labeled control DNA (Cat. No. 1585738)
Control Oligonucleotide, DIG-ddUTP-labeled	2.5 pmol/µl oligonucleotide, labeled with Digoxigenin-11-ddUTP according to the standard labeling procedure	• Vial 6, DIG Oligonucleotide 3'-End Labeling Kit • DIG-3'-End labeled control oligonucleotide (Cat. No. 1585754)
Control Oligonucleotide, DIG-dUTP/dATP tailed	2.5 pmol/µl oligonucleotide, tailed with Digoxigenin-11-dUTP and dATP according to the standard labeling procedure	• Vial 6, DIG Oligonucleotide Tailing Kit
Labeled Control RNA	Digoxigenin-labeled "antisense"-Neo RNA, transcribed with T7RNA polymerase from 1 µg template DNA, according to the standard labeling procedure. The solution contains approx. 100 µg/ml DIG-labeled RNA and 10 µg/ml unlabeled DNA template.	• Vial 5, DIG RNA Labeling Kit • DIG-labeled control RNA (Cat. No. 1585746)

In addition to the DIG-labeled control you will need the *Reagents* and the *Additionally required reagents*, listed above under "Estimating the yield with DIG Quantification and DIG Control Teststrips".

Procedure

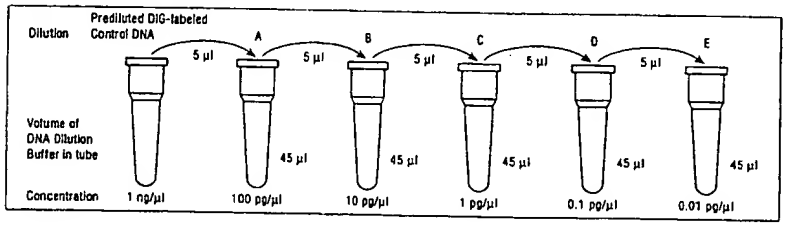
❶ Make a predilution of the DIG-labeled Control DNA by mixing 5 µl DIG-labeled Control DNA with 20 µl DNA dilution buffer (final concentration 1 ng/µl).
or
Make a predilution of the DIG-labeled Control RNA by mixing 5 µl DIG-labeled Control RNA with 20 µl DMPC-treated H_2O (final concentration 20 ng/µl).
For the DIG-3'-end labeled or tailed control oligonucleotide a predilution is not required.

❷ Make serial dilutions of the (prediluted) controls, according to the appropriate dilution scheme. Mix thoroughly between dilution steps.

Dilution Scheme A (for DNA probes) ▼

DIG-labeled Control DNA Starting Concentration	Stepwise Dilution	Final Concentration (dilution name)	Total Dilution
1 ng/µl	5 µl/45 µl DNA dilution buffer	100 pg/µl (A)	1:10
100 pg/µl (dilution A)	5 µl/45 µl DNA dilution buffer	10 pg/µl (B)	1:100
10 pg/µl (dilution B)	5 µl/45 µl DNA dilution buffer	1 pg/µl (C)	1:1,000
1 pg/µl (dilution C)	5 µl/45 µl DNA dilution buffer	0.1 pg/µl (D)	1:10,000
0.1 pg/µl (dilution D)	5 µl/45 µl DNA dilution buffer	0.01 pg/µl (E)	1:100,000

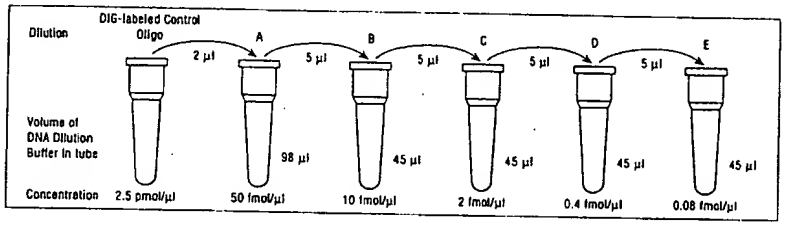
Dilutions A–E can be stored at –20°C for at least 1 year.



Dilution Scheme B (for Oligonucleotide probes) ▼

DIG-tailed- or end-labeled Control Oligo Starting Concentration	Stepwise Dilution	Final Concentration (dilution name)	Total Dilution
2.5 pmol/µl	2 µl/98 µl DNA dilution buffer	50 fmol/µl (A)	1:50
50 fmol/µl (dilution A)	10 µl/40 µl DNA dilution buffer	10 fmol/µl (B)	1:250
10 fmol/µl (dilution B)	10 µl/40 µl DNA dilution buffer	2 fmol/µl (C)	1:1,250
2 fmol/µl (dilution C)	10 µl/40 µl DNA dilution buffer	0.4 fmol/µl (D)	1:6,250
0.4 fmol/µl (dilution D)	10 µl/40 µl DNA dilution buffer	0.08 fmol/µl (E)	1:31,250

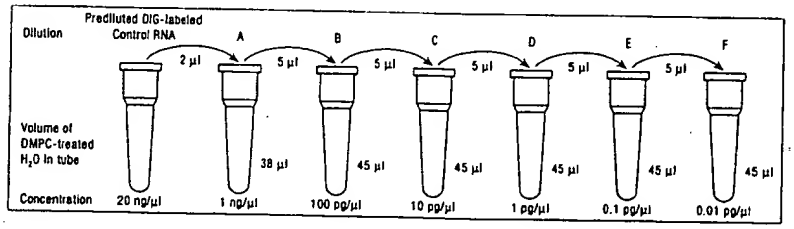
Dilutions A–E can be stored at –20°C for at least 1 year.



Dilution Scheme C (for RNA probes) ▼

DIG-labeled Control RNA Starting Concentration	Stepwise Dilution	Final Concentration (dilution name)	Total Dilution
20 ng/µl	2 µl/38 µl H_2O	1 ng/µl (A)	1:20
1 ng/µl (dilution A)	5 µl/45 µl H_2O	100 pg/µl (B)	1:200
100 pg/µl (dilution B)	5 µl/45 µl H_2O	10 pg/µl (C)	1:2,000
10 pg/µl (dilution C)	5 µl/45 µl H_2O	1 pg/µl (D)	1:20,000
1 pg/µl (dilution D)	5 µl/45 µl H_2O	0.1 pg/µl (E)	1:200,000
0.1 pg/µl (dilution E)	5 µl/45 µl H_2O	0.01 pg/µl (F)	1:2,000,000

Highly diluted solutions of RNA in H_2O are not very stable. Spots have to be made immediately after preparing the dilutions. Alternatively the RNA can be diluted in RNA dilution buffer (DMPC-treated H_2O, 20 x SSC and formaldehyde, mixed in a volume ratio of 5 + 3 + 2) for greater stability.



❶ Use the tables on page 17 or 18 to estimate the expected yield of DNA labeling reactions. Predilute an aliquot of the newly labeled experimental DNA probe to an expected final concentration of approx. 1 ng/µl.
or
Predilute an aliquot of the newly labeled experimental oligonucleotide probe to a final concentration of 2.5 pmol/µl.
or
Predilute an aliquot of the newly synthesized experimental RNA probe to an expected final concentration of approx. 20 ng/µl. In a standard RNA labeling reaction approx. 10 µg newly synthesized DIG-RNA probe is transcribed from 1 µg DNA template

❷ Make serial dilutions of the prediluted experimental probe, according to the appropriate dilution scheme:
- for DNA probes, use dilution scheme A,
- for oligonucleotide probes, use dilution scheme B,
- for RNA probes, use dilution scheme C.

❸ Spot 1 µl of the diluted controls on a piece of nylon membrane:
- for DNA probes, spot dilutions B–E,
- for oligonucleotide probes, spot dilution A–E,
- for RNA probes, spot dilutions C–F.

❹ In a second row, spot 1 µl of the corresponding dilutions of the experimental probe.

❺ Fix the nucleic acids to the membrane by cross-linking with UV-light or by baking for 30 min at +120°C (Boehringer Mannheim Nylon Membrane).

❻ Wash the membrane briefly in washing buffer.

❼ Incubate the membrane in blocking solution for 30 min at room temperature.

❽ Dilute Anti-DIG-alkaline phosphatase 1:5,000 in blocking solution.

❾ Incubate the membrane in the diluted antibody solution for 30 min at room temperature. The diluted antibody solution must cover the entire membrane.

❿ Wash the membrane twice, 15 min per wash, in washing buffer at room temperature.

⓫ Incubate the membrane in detection buffer for 2 min.

⓬ Mix 45 µl NBT solution and 35 µl BCIP solution in 10 ml of detection buffer. This color substrate solution must be prepared freshly.
Note: Alternatively, chemiluminescent detection can be performed, as described in the "Detection" section on page 58–60.

⓭ Pour off the detection buffer and add the color substrate solution. Allow the color development to occur in the dark. The color precipitate starts to form within a few minutes and continues for approx. 16 h.
Do not shake while the color is developing.

⓮ When the spots appear in sufficient intensity, stop the reaction by washing the membrane with TE buffer or sterile H_2O for 5 min.

⓯ Compare spot intensities of the control and experimental dilutions to estimate the concentration of the experimental probe (See Figure 6).

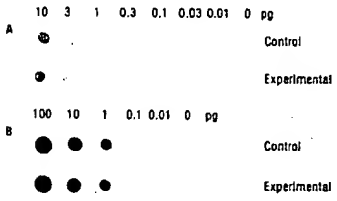


Figure 6: Estimating the Yield of DIG-labeled DNA. Dilutions of the Labeled Control DNA and the newly labeled (experimental) DNA were spotted on, fixed to, and directly detected on a Boehringer Mannheim Nylon Membrane, with colorimetric (Panel A) or chemiluminescent detection (Panel B).

What to do next

At this time, proceed to the "Hybridization" section of this User's Guide, which begins on page 42.

Chapter 6 • Purification of DIG-Labeled Nucleic Acids

For filter hybridizations, it is usually not necessary to clean up probe prior to hybridization. However, if you have a desire to clean up the probe or if you will be using it for *in situ* hybridization, we can recommend the following purification procedures;

- ethanol precipitation
- purification with the High Pure PCR Product Purification Kit.

Purification of the DIG-labeled DNA with the High Pure PCR Product Purification Kit is especially useful when the template DNA was isolated from agarose gels. Remaining agarose particles, that can be a source for background, are efficiently removed with this procedure.

Ethanol Precipitation

Products required

Name in procedure	Description	Available as
Glycogen solution	20 mg/ml glycogen in redistilled water	• Glycogen (Cat. No. 901 393)

Additionally required solutions

Additionally required solution	Description
LiCl	4 M lithium chloride solution; do not use ammonium acetate or sodium acetate
Ethanol	Absolute ethanol, chilled at –20°C; when 70% ethanol is indicated, dilute ethanol with redistilled water.
TE-buffer	10 mM Tris-HCl, 1 mM EDTA; pH 7.0–8.0

Procedure

1. Optionally: add 1 µl Glycogen solution to the reaction tube and mix thoroughly. *Note: We recommend the addition of glycogen for the precipitation of oligonucleotides after labeling with terminal transferase (DIG Oligonucleotide 3'-End Labeling Kit or DIG Oligonucleotide 3'-Tailing Kit). In general the addition of glycogen as carrier for precipitation is only necessary when low amounts of nucleic acids are to be precipitated.*
2. Precipitate the labeled nucleic acid with 0.1 volume of 4 M LiCl and 2.5–3.0 volumes of chilled ethanol. Mix well and incubate at –70°C for 30 min, or at –20°C overnight.
3. Centrifuge the reaction at 13,000 x g for 15 min in a microcentrifuge.
4. Decant the ethanol and wash the pellet with 100 µl of icecold 70% ethanol.
5. Centrifuge at 13,000 x g for 5 min in a microcentrifuge, then remove the ethanol.
6. Dry the pellet and resuspend in 50 µl of TE buffer. If not used immediately, store the labeled probe at –20°C (–70°C for RNA-probes).

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.

Purification with the High Pure PCR Product Purification Kit

The High Pure PCR Product Purification Kit is designed for the efficient and convenient isolation of PCR products from amplification reactions, but is also suited for the removal of unincorporated nucleotides from DIG DNA labeling reactions. The DNA binds specifically to the surface of glass fibres in the presence of chaotrope salts. Primers, unincorporated nucleotides, contaminating agarose particles and proteins are removed by a simple washing step. The bound DIG-labeled DNA is subsequently eluted in a low-salt buffer.

Note: A minimum length of approx. 100 bp is required for efficient binding. The kit can therefore not be used for the removal of unincorporated nucleotides from oligonucleotide labeling reactions.

Products required

Name in procedure	Description	Available as
High Pure PCR Product Purification Kit	Kit for 50 purifications Kit for 250 purifications	Cat. No. 1732 668 Cat. No. 1732 676
consisting of:		
• Binding buffer, green cap	nucleic acids binding buffer; 3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% (v/v) ethanol, pH 6.6 (25°C)	• Vial 1
• Wash buffer, blue cap	wash buffer; add 4 volumes of absolute ethanol before use! Final concentrations; 20 mM NaCl, 2 mM Tris-HCl, pH 7.5 (25°C), 80% ethanol	• Vial 2
• Elution buffer	elution buffer; 10 mM Tris-HCl, 1 mM EDTA, pH 8.5 (25°C)	• Vial 3
• High Pure filter tubes	Polypropylene tubes, containing two layers of a specially pre-treated glass fibre fleece; maximum sample volume: 700 µl	
• Collection tubes	2 ml polypropylene tubes	

Procedure

Note: Make sure that 4 volumes ethanol have been added to the wash buffer (vial 2, blue cap). The binding buffer (vial 1, green cap) contains guanidine-thiocyanate which is an irritant. Wear gloves and follow laboratory safety conditions during handling.

❶ Fill up the labeling reaction to 100 µl with redistilled water.

❷ Add 500 µl binding buffer (vial 1, green cap) and mix well.
Note: It is important that the volume ratio between sample and binding buffer is 1:5. When using other sample volumes than 100 µl, adjust the volume of binding buffer accordingly.

❸ Insert a High Pure filter in a collection tube and pipette the sample into the upper buffer reservoir.

❹ Centrifuge at 13.000 x g in a microcentrifuge for 30 sec.

❺ Discard the flow through and combine the filter tube again with the same collection tube.

❻ Add 500 µl wash buffer (vial 2, blue cap) to the upper reservoir and centrifuge as in step 4.

❼ Discard the wash buffer flow through and recombine the filter tube again with the same collection tube.

❽ Add 200 µl wash buffer (vial 2, blue cap) to the upper reservoir and centrifuge as in step 4.

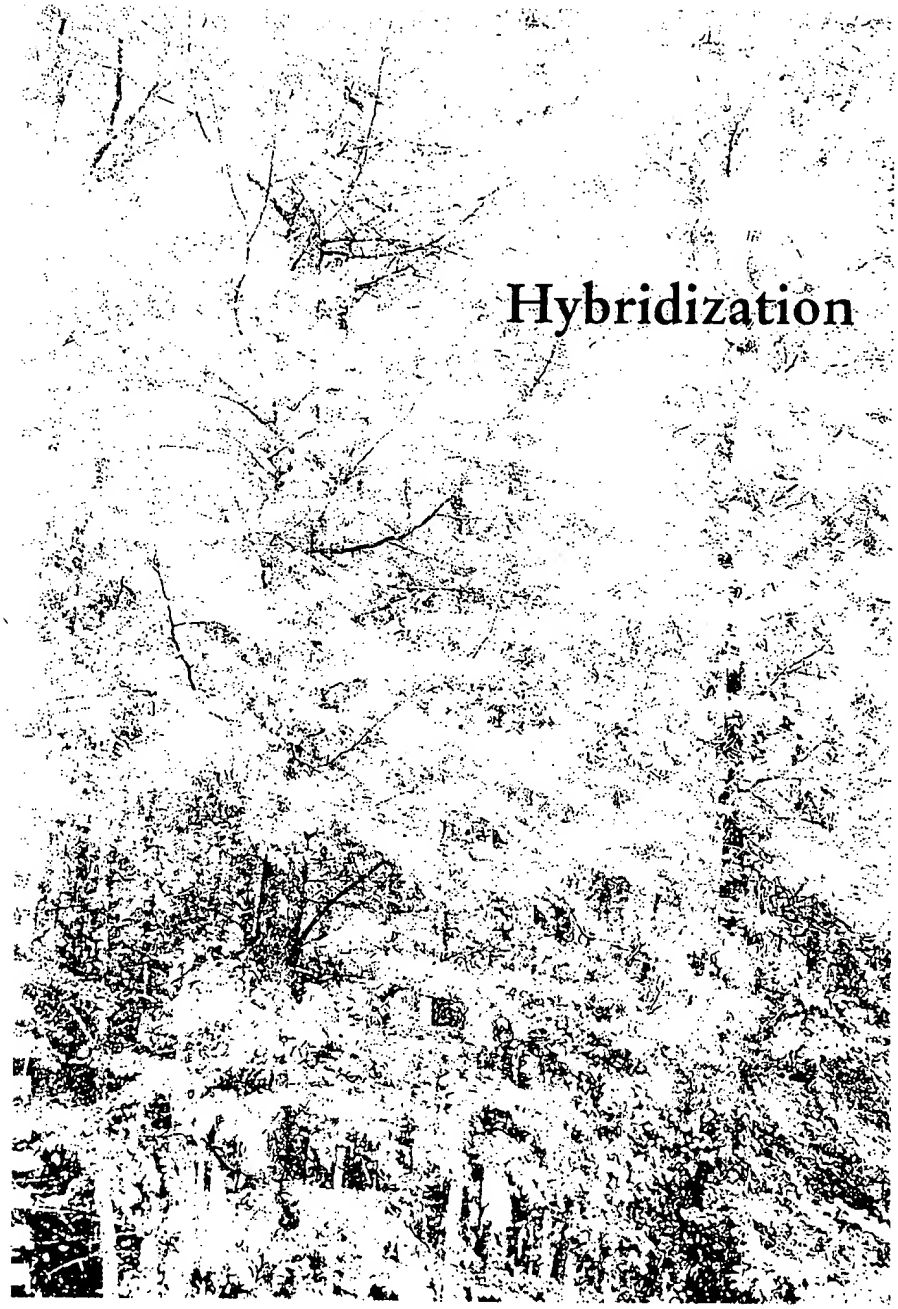
❾ Discard the collection tube and insert the filter tube in a clean 1.5 ml reaction tube (not provided).

❿ Add 50–100 µl elution buffer (vial 3) or redist. water (pH 8.0–8.5) to the upper reservoir for the elution of the DNA. Centrifuge as in step 4.

Note: The elution efficiency is increased with higher volume of elution buffer applied. At least 68% and 79% recovery are found with 50 and 100 µl elution buffer, respectively. Normally, almost quantitative recovery can be found, as can be determined in a direct detection assay.

What to do next

For all labeling reactions, it is extremely important that you verify labeling efficiency in a direct detection assay. Prior to hybridization, proceed to the "Estimating the Yield of DIG-labeled Nucleic Acids" protocol on page 33.


Hybridization

Chapter 7 • General Considerations for Hybridization

Please review this section of general hybridization considerations before proceeding with the DIG-system. Several points are critical for successful use of the DIG-system, especially when performing chemiluminescent detection. For general information on nucleic acid hybridization, see

▶ Sambrook, J., Fritsch, E. M. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Membrane Selection

For best results, use Boehringer Mannheim's „Nylon Membranes, positively charged (Cat. Nos. 1209299, 1209272, 1417240) for the transfer. This membrane has an optimal charge density, allowing it to bind the nucleic acid tightly without producing background. The nylon membranes are also specifically tested with the DIG-system to ensure optimal signal-to-noise ratios.

Other, uncharged membranes can also be used with the DIG-system. Their binding capacity is lower and therefore a lower maximum sensitivity can be achieved. In general, lower background can be expected when using uncharged nylon membranes. They are, however, not tested in combination with the DIG-System.

Nitrocellulose membranes cannot be recommended in combination with the DIG-System. They can only be used when colorimetric detection will be performed and no stripping and reprobing is planned.

Probe Concentration

In the following chapters we give recommendations for probe concentrations in the different applications. These recommendation refer to newly sythesized, DIG-labeled probe. In the package inserts of the respective labeling kits and in the „Labeling"-section of this guide, an expected yield for labeling under standard conditions is given. This must however be confirmed by estimating the yield of a labeling reaction, as is described in Chapter 5, page 33.

The recommended probe concentration must be regarded as a starting point for your hybridization. For the most accurate determination of optimal probe concentration we recommend to perform a mock hybridization (described below).

Note: If chemiluminescent detection is performed, a too high probe concentration will often lead to background. Therefore the probe concentration should not be increased above the recommended concentrations. When the chemiluminescent substrate CDP-Star™ is used, you will generally need lower probe concentrations than with chemiluminescent detection with CSPD®.

Optimization of the probe concentration – the "mock" hybridization

To prevent background problems as a result of a too high probe concentration, we recommend to optimize the probe concentration in a mock hybridization, before the actual hybridization is performed.

The mock hybridization is carried out by incubating small membrane pieces (without DNA transferred to it) with different probe concentrations in the hybridization solution and subsequent detection with the procedure of choice.
▼
For example

Probe type	Concentration in the hybridization solution		
DNA/RNA probes	1 µl*/ml	3 µl*/ml	5 µl*/ml
End-labeled oligonucleotide	1 pmol/ml	3 pmol/ml	10 pmol/ml
Tailed oligonucleotide	0.1 pmol/ml	0.5 pmol/ml	2 pmol/ml

* from the labeling reaction

The highest probe concentration that gives an acceptable background should be used for the hybridization experiment (see figure 7, 25 ng/ml).

Probe Filtration

Small particles that are contaminating the probe can be filtered out through a 0.45 µm filter. This can be performed best after addition of the probe to prewarmed (to hybridization temperature) DIG Easy Hyb and filtration of this entire hybridization solution (for information on hybridization buffers, see below). The filtration results in lower spot-like background (see figure 7). *Note: This can only be performed when DIG Easy Hyb is used as hybridization buffer. Other hybridization buffers have components (e.g. Blocking Reagent) that cannot be filtered through a 0.45 µm filter. When you want to use another hybridization buffer and want to purify the probe, we recommend to use the procedure with High Pure PCR Product Purification Kit, described on page 40.*

Labeled probes can hybridize non-specifically to sequences that bear homology but are not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matched hybrids. They can be dissociated by performing washes of various stringency. The stringency of washes can be manipulated by varying the salt concentration and temperature. For some applications, the stringency of the washes should be higher. However we recommend that you hybridize stringently (i.e., optimize hybridization conditions) rather than wash stringently.

Prehybridization/Hybridization solutions

Several hybridization buffers can be used with the DIG-System. In our experience, optimal results have been obtained with the buffers, listed page 44. The main difference with hybridization buffers described elsewhere, is the presence of Blocking Reagent. The protein in Blocking Reagent reduces the non-specific binding of probe to the membrane filter.

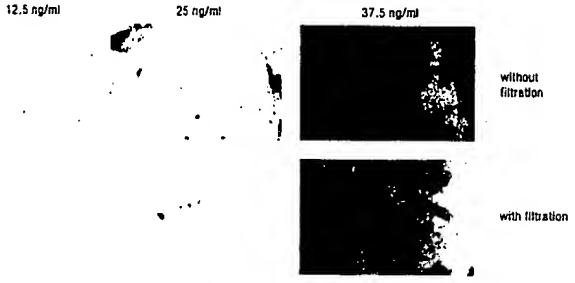


Figure 7: Mock hybridization and effect of probe filtration. Naked pieces of membrane were incubated with the indicated amounts of DIG-labeled DNA probe, with and without filtration through 0.45 µm filters, and detected with chemiluminescence.

Hybridization and Washing Conditions

We have found that DIG-labeled probes demonstrate the same hybridization kinetics as radiolabeled probes. Hybridization and washing conditions for DIG-labeled probes do not differ substantially from those of radiolabeled probes. The optimal hybridization and wash conditions for each probe must be determined experimentally. In this User's Guide, we provide recommendations for hybridization and washing conditions. Use the conditions given as a starting point. It may then be necessary to optimize conditions to obtain maximum sensitivity with your probe.

DIG Easy Hyb*	Standard buffer	Standard buffer + 50% formamide	High SDS buffer (Church buffer)
Cat. No. 1603558 500 ml, ready-to-use solution	5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 1% Blocking Reagent	50% formamide, deionized, 5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02 (w/v) SDS, 2% Blocking Reagent	7% SDS, 50% formamide, deionized, 5 x SSC, 2% Blocking Reagent, 50 mM sodium phosphate, pH 7.0, 0.1% (w/v) N-lauroylsarcosine

▲ **Table 6: Composition of recommended hybridization solutions**

*DIG Easy Hyb is a ready-to-use hybridization buffer. It is a non-toxic solution that can be used like a formamide-based hybridization buffer and is highly recommended for all membrane based applications.

Hybridization containers

You may use sealable containers or heat sealable plastic bags for the hybridization. The prehybridization and washings are generally performed in sample volume; here the membrane must be allowed to float freely, e.g. in a clean tray.

Roller tubes in combination with a hybridization oven may also be used. Use at least 6 ml of (pre-)hybridization solution per tube.

Note: The hybridization temperature should be monitored inside the roller tube. There might be a difference in the adjusted temperature and the temperature inside the bottle. Check the temperature by filling a tube with water and placing a thermometer inside the tube.

Storage and Reuse of Hybridization Solutions

One of the advantages of the DIG-System is the stability of the labeled probe. After hybridization against the blotted target, the hybridization solution still contains large amounts of unannealed DIG-labeled probe. Simply pour the solution into a plastic tube and store at –20°C for DNA probes and –70°C for RNA probes. DIG labeled probes are stable for at least 1 year when stored in this manner.

For reuse, thaw and denature by heating to +95°C for 10 min. If the hybridization solution contains formamide or if DIG Easy Hyb was used, denature at 68°C for 10 min.

Stripping and reprobing

With the DIG-System, membranes can be stripped and reprobed. To do this refer to the procedures, described on page 66.
Note: When reprobing is planned, membranes must be kept wet at all stages, after the first probe has been applied.

What to do next

At this time, proceed to the appropriate application in the "Hybridization" section of the User's Guide.

Chapter 8 • Hybridization Techniques

Southern Blotting

The DIG-System can detect 0.03 pg (chemiluminescent detection) or 0.1 pg (colorimetric detection) homologous DNA in a Southern blot format on a nylon membrane. This corresponds to the detection of a single-copy gene in < 1 µg of human genomic DNA. The procedures described here, are used routinely in our labs and have been found to give optimal results in Southern blotting, particularly in genomic Southern blotting.

Required solutions

Solutions required for Southern blotting are listed below. Refer to Appendix B for details on preparing these required solutions.

Required solution	Description
HCl	250 mM HCl
H_2O	Sterile, distilled water
Denaturation solution 1	0.5 N NaOH, 1.5 m NaCl
Neutralization solution 1	0.5 M Tris-HCl, pH 7.5; 3 M NaCl
20 x SSC buffer	3 M NaCl, 300 mM sodium citrate, pH 7.0
5 x SSC buffer	750 mM NaCl, 75 mM sodium citrate, pH 7.0
Prehybridization solution	Prepare one of the following (see Table 7 for composition and Appendix B for details on preparation) • DIG Easy Hyb • Standard buffer • Standard buffer + 50% formamide • High SDS buffer
Hybridization solution	DIG-labeled probe, diluted in prehybridization solution
2 x Wash solution	2 x SSC, containing 0.1% SDS
0.5 x Wash solution	0.5 x SSC, containing 0.1% SDS

Gel Electrophoresis

Restriction digest the DNA. Prepare an agarose gel of appropriate percentage, using a high-purity, nucleic acid grade agarose, such as Agarose MP or Agarose LE (available from Boehringer Mannheim), and Tris-Borate-EDTA (TBE)- or Tris-Acetate-EDTA (TAE)-buffer. Run the digest on the gel. If desired, the gel may be stained with ethidium bromide to visualize DNA fragments and to confirm the subsequent transfer to the membrane.

Southern Transfer

The transfer of DNA from the gel to the membrane can be accomplished by one of a number of common procedures; however the following procedures are routinely used in our lab and provide optimal detection sensitivity.

Depurination (optional)

Controlled acid treatment depurinates DNA. In the subsequent alkaline denaturation of the DNA, the DNA-strand breaks at the depurinated sites, resulting in smaller, easier to transfer fragments. Depurination is an optional treatment, usually performed when fragments >10 kb must be transferred. If you are transferring small DNAs (<10 kb) or detecting only the smaller fragments in a genomic digest, it may not be necessary to depurinate the DNA. Avoid excessive acid treatment; the fragments will be too small, which results in poor detection sensitivity.

Procedure

1. Submerge the gel in 250 mM HCl for 10 min, with shaking, at room temperature. Do not exceed 10 min.
2. Rinse the gel with H_2O before proceeding to the "Denaturation section".

Denaturation, neutralization, and blotting

1. Submerge the gel in denaturation solution for 2 x 15 min at room temperature. Shake gently. *This treatment denatures the DNA, making it single-stranded and accessible for the later applied probe.*
2. Rinse the gel with H_2O.
3. Submerge the gel in neutralization solution for 2 x 15 min at room temperature.
4. Prepare membrane filters for Southern transfer, according to the manufacturer's recommendations. Boehringer Mannheim Nylon Membranes can be used without any prior treatments. Always use unpowdered rubber gloves when handling membranes, and manipulate the membranes with forceps at the edges only.
5. Check pH. This is especially necessary when working with nitrocellulose. The pH should be <9, but nylon membranes also tolerate a higher pH.
6. Blot the DNA from the gel by capillary transfer to the membrane, using 20x SSC buffer. Blot overnight to ensure efficient transfer of the DNA. Alternatively, the DNA can be vacuum-blotted onto the membrane; vacuum blotting can be accomplished in 1–2 h, according to the manufacturer's recommendations. Our experience indicates that capillary transfer is more efficient than vacuum transfer.

Fixation

Crosslink the DNA to the membrane by any of the following procedures.

- UV-crosslink the wet membrane without prior washing. After the UV crosslinking, rinse the membrane briefly in H_2O and allow to air-dry. *For UV-crosslinking of membranes special devices are available, that perform better than transilluminators.*

or

- Bake the membrane (Boehringer Mannheim Nylon Membranes) at +120°C for 30 min or according to the manufacturer's instructions.
- Nitrocellulose membranes must be baked at +80°C and under vacuum, to prevent spontaneous combustion of the nitrocellulose.

The membrane can now be used immediately for prehybridization, or can be stored dry at +4°C for future use.

Denaturation, neutralization, and blotting

1. Submerge the agarose gel in denaturation solution twice for 15 min at room temperature. Shake gently. This incubation denatures the DNA target prior to transfer.
2. Rinse the gel with H_2O.
3. Submerge the gel in neutralization solution twice for 15 min at room temperature to neutralize the gel.
4. Prepare membrane filters for Southern transfer according to the manufacturer's recommendations. *Note: Always use unpowdered rubber gloves when handling membranes, and manipulate the membrane with forceps on the membrane's edges.*
5. Especially when DNA transfer to nitrocellulose membranes is intended, it is important to check the actual pH of the gel after neutralization. It should be below pH 9 (nylon membranes will tolerate a higher pH) otherwise membranes will turn yellow and break during hybridization. To check the pH of the gel, lift one edge of the gel where no DNA has been loaded, press a pH stick into it and read the pH.
6. Blot the DNA from the gel by capillary transfer to the membrane, using 20 x SSC buffer. Blot overnight to ensure efficient transfer of the DNA. Alternatively, the DNA can be vacuum-blotted onto the membrane; vacuum-blotting can be accomplished in 1–2 h, according to the manufacturer's recommendations. Our experience indicates that capillary blotting is more efficient at transferring DNA than vacuum blotting.

DNA fixation

DNA can be efficiently bound to the nylon membrane by one of the following procedures.

Procedure

1. UV-crosslink the wet membrane without prior washing. After the UV-crosslinking, rinse the membrane briefly in H_2O and allow to air-dry.
2. Alternatively, the DNA can be fixed to the membrane by baking. Bake in an oven at +120°C for 30 min (Boehringer Mannheim Nylon Membranes).
3. Nitrocellulose membranes must be baked at 80°C under vacuum to prevent spontaneous combustion of the nitrocellulose.

The membrane can now be used immediately for prehybridization, or can be stored dry at +4°C for future use.

Prehybridization and Hybridization

Prehybridization prepares the membrane for probe hybridization by blocking nonspecific nucleic acid-binding sites on the membrane. This ultimately serves to lower background. Many different prehybridization solutions have been described in the literature. However, the prehybridization solutions described here combine efficient blocking with ease of use.

As with any probe, optimal hybridization conditions for DIG-labeled probes must be determined experimentally. We strongly recommend that the time be taken to optimize each DIG-labeled probe (see the mock hybridization on page 42). The time taken for optimization will result in cleaner results and, ultimately, time savings, especially if a probe will be reused many times.

Procedure

❶ Place the blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the bag, and prehybridize at the anticipated hybridization temperature for 2 h. Longer prehybridization times are acceptable. Several membranes can be processed in the same sealed bag as long as there is sufficient prehybridization solution to cover all the membranes, and the membranes can move freely in the bag.

The optimal hybridization temperature for a specific probe will depend on the length of the probe and on the extent of sequence homology with the target sequence; therefore, it must be determined empirically. See Table 7 for recommended temperatures for different types of probes and different hybridization solutions.

▼

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization*
DNA	5–25 ng/ml**	DIG Easy Hyb	Hybridize overnight at 37–42°C
		Standard buffer	Hybridize overnight at 65–68°C
		Standard buffer + 50% formamide	Hybridize overnight at 37–42°C
		High SDS buffer	Hybridize overnight at 37–42°C
RNA	100 ng/ml**	DIG Easy Hyb	Hybridize overnight at 50°C
		Standard buffer + 50% formamide	Hybridize overnight at 50°C
Oligonucleotides		DIG Easy Hyb	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. Sum up 4°C for each G or C and 2°C for each A or T. Perform prehybridization and hybridization at 10°C below the obtained T_m. Hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly (A) (in the prehybridization and hybridization solution) to prevent nonspecific hybridization signals. Additionally, 5 µg/ml of Poly (dA) may be added for further blocking.
tailed	0.1–2 pmol/ml	Standard buffer	
end-labeled	1–10 pmol/ml		

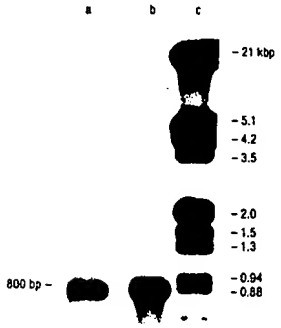
▲

Table 7: Optimal hybridization conditions for different probe types.

*The conditions given here are stringent conditions applicable if probe and target have 100% homology and a GC content of about 50%.

**When CDP-Star is used for detection the recommended concentrations are 10–20 ng/ml DIG-labeled DNA or 20–50 ng/ml DIG-labeled RNA. Higher concentrations may cause background.

❸ When using double-stranded DNA probes, heat in a boiling water bath for 10 min to denature the DNA. Chill directly on ice.
Single-stranded RNA probes and oligonucleotide probes do not require denaturation prior to dilution unless extensive secondary structure is predicted from the sequence. Prepare at least 3.5 ml hybridization solution for a blot of 10 x 10 cm.



DIG label
0.5 h exposure time
24 h Lumigen™ PPD

Figure 8: A typical Southern blot. Southern blot analysis of 10 µg *Hind* III-digested plant genomic DNA of transgenic tobacco ST1a containing a single copy of the *npt*-II gene (gift from M. Saul, personal communication), which was obtained by PEG-mediated direct gene transfer (M. Saul, et al., 1988). The DNA was transferred to the positively charged Nylon Membrane from Boehringer Mannheim and hybridized with a DIG-11-dUTP-labeled *Hind* III fragment of the plasmid pSHI 913 (M. Schnorf, et al., 1991) at a concentration of 25 ng DIG-labeled DNA/ml hybridization solution. The hybridization was performed in a hybridization oven in the presence of 50% formamide as described by Neuhaus-Url and Neuhaus.

A: 10 µg of *Hind* III-restricted plant DNA of ST1a releasing 1 copy of the 800 bp *npt*-II coding region.
B: 10 pg of the *Hind* III fragment of pSHI 913 reflecting 1 gene copy.
C: 40 ng of DIG-labeled Molecular Weight Marker III (Boehringer Mannheim).

Exposure time to X-ray film, to record the chemiluminescent signal was 0.5 h. The time elapsed between preincubation with the chemiluminescence substrate and exposure to X-ray film was 24 h.

References

1. Neuhaus-Url, G. and Neuhaus, G. (1993) *Transgen. Res.* 2, 115–120.
2. Saul, M. W., Shillito, R. D. and Negrutiu, I. (1988). In: Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. P. Verma (Eds.). Kluwer, Dordrecht, The Netherlands, pp 1–16.
3. Schnorf, M., Neuhaus-Url, G., Galli, A., Ilda, S., Potrykus, I. and Neuhaus, G. (1991) *Transgen. Res.* 1, 23–30.

Data were kindly provided by Dr. G. Neuhaus-Url, ETH, Zurich, Switzerland.

❹ Dilute the probe in hybridization solution. See Table 7 for optimal probe concentrations.
❹ Discard the prehybridization solution from the bag. Add the hybridization solution containing the DIG-labeled probe. Allow the probe to hybridize. See Table 7 for selecting a hybridization solution and temperature.
❺ At the end of the hybridization, pour the hybridization solution from the bag into a tube (with cap) that can withstand freezing and boiling (e.g., 50 ml polypropylene).
This hybridization solution contains unannealed DIG-labeled probe. The entire solution can be reused in future hybridization experiments. Label and date the tube, and store DNA probe solutions at –20°C and RNA probe solutions at –70°C. DIG-labeled probes stored in this manner are stable for at least 1 year. For reuse, thaw and denature by heating to +95°C for 10 min. If the hybridization solution contains 50% formamide (the flash point of pure formamide is +68°C) or DIG Easy Hyb, denature at +68°C for 10 min.
❻ Wash the membrane twice, 5 min per wash, in 2 x wash solution at room temperature. These washes (steps 6 and 7) remove unbound probe, which will lead to high backgrounds if not removed.
❼ Wash the membrane twice, 15 min per wash, in 0.5 x wash solution. Long probes (>100 bp) should be washed at 68°C. For shorter probes, the wash temperature must be determined empirically.
Note: For most applications, washing in 0.5 x wash solution is stringent enough. It must be determined empirically whether it is necessary to wash with 0.1 x wash solution (0.1 x SSC, containing 0.1% SDS.

What to do next

At this time, proceed to the "Detection" division of this User's Guide, which begins on page 58.

DNA Dot Blotting

Dot blots and slot blots are rapid methods for the qualitative screening of DNA. Target DNA samples may be purified DNA, cell lysate, or PCR-amplified DNA.

The same hybridization and detection procedures used with Southern blots are also performed on DNA dot blots; therefore, proceed to the Southern prehybridization and hybridization procedures (page 47) after preparing the dot blot.

Required solutions

Products and solutions that are required for the hybridization of DNA, but not specifically required for the dot blotting procedure given here, are listed in the Southern blotting application (page 45).

Required solution	Description
DNA dilution buffer	50 µg/ml herring sperm DNA; 10 mM Tris/HCl; pH 8.0; 1 mM EDTA, pH 8.0

Procedure

1. Prepare a dilution series of your DNA target in suitable amounts.
2. Denature the DNA target in the dilutions for 10 min at +95°C, and chill immediately on ice.
3. Mark the membrane lightly with a pencil to identify each dilution before spotting. We recommend Boehringer Mannheim Nylon Membranes, positively charged.
4. Dispense 1 µl of each dilution onto the membrane. Mix dilutions well before dotting on membrane.
5. Fix the DNA to the membrane by UV crosslinking or baking in an oven at +120°C for 30 min (Boehringer Mannheim Nylon Membranes).

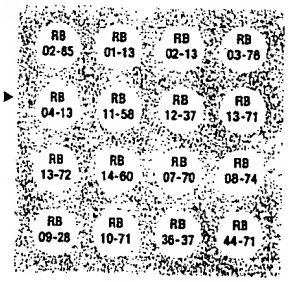


Figure 10: HLA-DR genotyping by chemiluminescent reverse Dot Blot. Sixteen sequence-specific oligonucleotides (SSOs) were blotted onto a nylon membrane. PCR-amplified target DNA (HLA-DR gene, second exons) corresponding to one individual was 3'-labeled with DIG-11-ddUTP by Terminal transferase, and hybridized to the immobilized SSOs. After washing, chemiluminescent detection was performed. The HLA-DR genotyping of this individual was found to be HLADRB1*01-DRB1*07. Data were kindly provided by Dr. J.F. Eliaou – Laboratoire d'Immunologie, Montpellier, France.

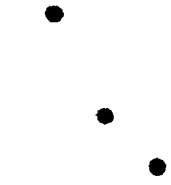


Figure 9: Chemiluminescent Dot Blot showing HLA class II typing. Human genomic DNAs from 48 patients were PCR amplified and blotted onto a nylon membrane. A HLA DRB1 01 sequence-specific oligonucleotide was 3'-end labeled with DIG-11-dUTP by Terminal transferase and hybridized to the membrane. After washing, chemiluminescent detection was performed. The blot was exposed to X-ray film for 15 min. Data were kindly provided by Dr. A. Moine-Grenoble Transfusion, La Tronche, France.

What to do next

Hybridize the samples on the dot blot according to the "Prehybridization and Hybridization" procedure described in the "Southern Blotting" application, which begins on page 45.

Colony and Plaque Hybridization

The DIG-System provides a sensitive and rapid method for detecting positive colonies or plaques in a heterologous background. Colony and plaque hybridizations have been developed to allow rapid screening of bacterial and phage recombinant libraries for specific DNA sequences. The bacterial colonies or phage particles are transferred to a nylon membrane. Alkaline treatment serves to lyse the colonies or to disassemble the phage particles. The denatured DNA is then immobilized on the membrane, followed by a proteinase K treatment to digest interfering proteins. A digoxigenin-labeled DNA, RNA or oligonucleotide probe is used for hybridization. Detection is carried out with a colorimetric or chemiluminescent immunoassay.

Recommended Membranes

We recommend to use Nylon Membranes for Colony and Plaque Hybridization, Cat. Nos. 1699075 (Ø 82 mm) and 1699083 (Ø 132 mm). The membranes are uncharged at pH 6.5 and have a pore size of 1.2 µm. The membrane discs are especially suited and tested for nonradioactive screening of phage or cosmid libraries with DIG-labeled probes and detection with highly sensitive chemiluminescent (CSPD, CDP-Star) or chromogenic substrates (NBT/BCIP, Multicolor Detection Set). The optimized retention of nucleic acids and the mechanical strength allow multiple stripping and reprobing with different probes.

Fixation of the DNA to the Nylon Membranes for Colony and Plaque Hybridization can be performed by UV crosslinking or by baking at + 80°C.

Required Solutions

Refer to Appendix B for details on preparing these additionally required solutions.

Required solution	Description
Denaturation solution 1	0.5 N NaOH, 1.5 m NaCl
Neutralization solution 2	1.0 M Tris-HCl, pH 7.5; 1.5 M NaCl
20 x SSC buffer	3 M NaCl, 300 mM sodium citrate, pH 7.0
2 x SSC buffer	0.3 mM NaCl, 30 mM sodium citrate, pH 7.0
Proteinase K	2 mg/ml Proteinase K in 2 x SSC buffer dilute Proteinase K (> 600 U/ml, 14–22 mg/ml; Cat. Nos. 1413783, 1373196, 1373200) 1 to 10 in 2 x SSC) *or* dissolve Proteinase K (lyophilisate, approx. 20 U/mg; Cat. Nos. 161519, 745723, 1000144, 1092766) 2 mg/ml in 2 x SSC
Prehybridization solution	Prepare one of the following (see Table 8 for composition and Appendix B for details on preparation) • DIG Easy Hyb • Standard buffer • Standard buffer + 50% formamide
Hybridization solution	DIG-labeled probe, diluted in prehybridization solution
2 x Wash solution	2 x SSC, containing 0.1% SDS
0.5 x Wash solution	0.5 x SSC, containing 0.1% SDS

Any type of DIG-labeled DNA, RNA or oligonucleotide probe can be used for colony and plaque hybridization. However, to avoid nonspecific hybridization, use a probe that does not contain any sequences homologous to the vector. For DNA probes it is therefore recommended to use isolated inserts as template for the probe labeling.

The optimal hybridization temperature and probe concentration must be determined empirically. Table 8 offers general guidelines. ▼

Table 8: Hybridization conditions for different probe types

Probe type	Probe concentration	Hybridization solution	Time and temperature for prehybridization and hybridization*
DNA	5–25 ng/ml**	DIG Easy Hyb	Hybridize for 2 h at +42°C
		Standard buffer + 50% formamide	Hybridize overnight at +42°C
		Standard buffer	Hybridize overnight at +68°C
RNA	100 ng/ml**	DIG Easy Hyb	Hybridize for 2 h at +50°C
		Standard buffer + 50% formamide	Hybridize overnight at +50°C
Oligonucleotides tailed	0.1–2 pmol/ml	DIG Easy Hyb	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. (To estimate the T_m, add 4°C for each G or C and 2°C for each A or T. Hybridize at 10°C below this estimated T_m). To prevent non-specific hybridization signals, hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly (A) in the prehybridization and hybridization solution. Additionally, 5 µg/ml of Poly (dA) may be added for further blocking.
end-labeled	1–10 pmol/ml	Standard buffer	

*The conditions given here are stringent conditions, applicable when probe and target have 100% homology and a G plus C content of about 50%.

**When CDP-Star is used for detection, the recommended probe concentrations are 10–20 ng/ml DIG-labeled DNA or 20–50 ng/ml DIG-labeled RNA. Higher concentrations may cause background.

Procedure

Colony/plaque lifts

❶ Soak 2 layers of Whatman 3MM paper for each different solution: denaturation solution, neutralization solution and 2 x SSC.

❷ Pre-cool colonies or plaques on agarose plates for approx. 30 min at 4°C. For plaque lifts we recommend the use of Agarose MP (Cat. Nos. 1444964, 1388983, 1388991) for the top agar (0.7% in YT-medium).

❸ Carefully place a membrane disc onto the surface. Avoid air bubbles. Do not move the membrane once it has been applied, as transfer begins almost immediately.

❹ Leave the membrane on the plate for approx. 1 min. Mark the orientation of the membrane to the plate, in order to be able to identify the positive colonies or plaques after detection.

❺ Remove membrane disc carefully with filter tweezers and blot briefly (colonies/plaques-side up) on dry Whatman 3MM paper.

❻ Place membrane discs (colonies/plaques-side up) for 15 min (colony lifts) or 5 min (plaque lifts) on the prepared filter paper soaked with denaturation solution.

❼ Blot briefly on Whatman 3MM paper.

❽ Place membrane discs (colonies/plaques-side up) for 15 min onto the prepared filter paper soaked with neutralization solution.

❾ Blot briefly on Whatman 3MM paper.

❿ Place membrane discs for 10 min onto the prepared filter paper soaked with 2 x SSC.

⓫ Crosslink the transferred DNA with UV-light or by baking the dry membranes for at least 30 min at 80°C.

Proteinase K treatment

❶ Place membrane discs on a clean piece of aluminium foil and pipet 0.5 ml of 2 mg/ml Proteinase K on each membrane disc (0.5 ml for the discs Ø 82 mm).

❷ Distribute the solution evenly, incubate for 1 h at 37°C.

❸ Using filter paper fully wetted with dH_2O, blot membranes between the filter paper, and apply pressure by passing over the area with a ruler or a bottle.

❹ Remove cellular and agar debris by gently pulling off the upper filter paper (the debris will stick to this filter paper). Check the complete removal of all cellular debris. When necessary repeat the blotting step with a fresh piece of filter paper, soaked in water. The filters are now ready for hybridization.

Hybridization

The membranes can be hybridized in roller bottles, glass dishes, or sealed in hybridization bags. Make sure that the membranes do not stick together and are sufficiently covered with hybridization solution. We recommend no more than 4 to 5 membranes per hybridization vessel, or up to 3 membranes per roller bottle.

The volumes in the following protocol are calculated for the use of 275 ml roller bottles.

1. Place up to 3 membrane discs (Ø 82 mm) in a roller bottle, add 60 ml prehybridization solution.
2. Prehybridize for 1 h in a hybridization oven at the recommended temperature (see Table 8)
3. Denature the labeled probe (double stranded probes only) by boiling for 5 min at 95–100°C. Rapidly cool on ice.
4. Mix the denatured probe with 6 ml hybridization solution, prewarmed to hybridization temperature.
5. Remove the prehybridization solution and add the hybridization solution.
6. Incubate according to the recommendations in Table 8.
7. At the end of the hybridization, pour the hybridization solution into a tube that can withstand freezing and boiling (e.g. a 50 ml polypropylene tube). *The hybridization solution can be reused several times, as long as the probe has not been depleted from solution. The DIG-labeled probes are stable for at least 1 year when stored at –20°C (DNA and oligonucleotide probes) or –70°C (RNA probes). For reuse, thaw and denature the entire mix by heating to 95°C for 10 min. When DIG Easy Hyb is used or when the hybridization solution contains formamide, denature at +68°C for 10 min.*

Stringency washes

1. Wash the membranes twice for 5 min in ample 2 x SSC, 0.1% SDS min at room temperature with gentle agitation.
2. Transfer the membranes to 0.5 x SSC, 0.1% SDS and wash twice for 15 min at 68°C with gentle agitation.

Note on subsequent detection

▶ Besides chemiluminescent detection or colorimetric detection with NBT/BCIP, we also recommend the use of the Multicolor Detection Set for detection. This detection method, described on page 62, allows the simultaneous detection of three different probe/target hybrids on a single filter.

What to do next

At this time, proceed to the "Detection" division of this User's Guide, which begins on page 58.

Northern Blotting

The DIG System can be readily used to detect RNA on a membrane. The results that are obtained with the DIG System are equivalent to those achieved with radioactive techniques. The same parameters in choosing hybridization conditions apply to both systems.

Probe Preparation

As is the case with radioactive probes, DIG-labeled RNA probes demonstrate stronger signals and less non-specific hybridization than DNA probes on Northern and Southern Blots. Therefore we recommend to use a RNA probe whenever possible.

If a DNA probe must be used, we recommend that you use the high SDS hybridization buffer or DIG Easy Hyb to reduce background. See Table 9 for details on hybridization solutions for different probe types.

Optimization of the Probe Concentration

Optimize the probe concentration before all hybridization experiments. This is necessary to avoid background staining, and it can be easily performed with a series of mock hybridization, where increasing concentrations of DIG-labeled probes are incubated with naked pieces of membrane or hybridized to dots of homologous DNA or RNA. This procedure is described on page 42.

Avoidance of RNase Contamination

Throughout the northern blot experiment, be careful to avoid the introduction of RNases, as RNA is susceptible to degradation even after its immobilization on a nylon membrane. We recommend sterilization of all solutions and containers that will come in contact with the RNA or northern blot. In addition to autoclaving, treat solutions and containers with DMPC (dimethylpyrocarbonate) or DEPC (diethylpyrocarbonate).

Throughout the experiment, use forceps whenever possible, and wear gloves.

Optimal Blotting Conditions

Salt concentrations between 10 x and 20 x SSC give equivalent results for the transfer of RNA from a 1% agarose formaldehyde gel to a nylon membrane. The optimal blotting duration is overnight at 4°C or room temperature.

Required solutions

Required solution	Description
Prehybridization solution	Prepare one of the following (see Table 9 for hybridization solution requirements, and see Appendix B for details on preparation) • DIG Easy Hyb • High SDS buffer • Standard buffer + 50% formamide
Hybridization solution	DIG-labeled probe, diluted in prehybridization solution
2 x Wash solution	2 x SSC, containing 0.1% SDS
0.5 x Wash solution	0.5 x SSC, containing 0.1% SDS
20 x SSC	3 M NaCl, 300 mM sodium citrate, pH 7.0; treated with DMPC
10 x SSC	1.5 M NaCl, 150 mM sodium citrate, pH 7.0 treated with DMPC

Controls

A DIG-labeled anti-sense Actin RNA hybridization probe (Cat. No. 1498045) is available for evaluating the quality and quantity of your RNA.

Northern Transfer

1. After electrophoresis in a standard formaldehyde gel, equilibrate the gel in 20 x SSC (DMPC-treated) for 2 x 15 min.
2. Prepare a membrane filter. Wear powder-free gloves when handling the membrane, and manipulate the membrane by applying forceps to the edges.
   For best results, use Boehringer Mannheim's Nylon Membranes (Cat. Nos. 1209299, 1209272, 1417240) for the transfer. This membrane has an optimal charge density, allowing it to bind the RNA tightly without producing high backgrounds. Our nylon membrane is also specifically tested with the DIG System to ensure optimal background characteristics.
3. Blot the RNA from the gel by capillary transfer overnight at +4°C for 4 h at room temperature with 10 x or 20 x SSC (DMPC-treated).
4. UV-crosslink or bake the membrane at +120°C for 30 min.

Prehybridization and Hybridization

Before hybridization, determine the optimal probe concentration according to the mock hybridization protocol on page 42. Table 9 gives general guidelines for probe concentrations and hybridization temperatures.

❶ Place the blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the bag, and prehybridize at the anticipated hybridization temperature for at least 1 h.
Longer prehybridization times are acceptable.

Table 9: Optimal hybridization conditions for different probe types.

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization*
RNA	50–100 ng/ml**	DIG Easy Hyb	Hybridize overnight at 68°C
		Standard buffer + 50% formamide	Hybridize overnight at 68°C
DNA	25 ng/ml**	DIG Easy Hyb	Hybridize overnight at +50°C
		High SDS buffer	Hybridize overnight at +50°C
Oligonucleotides		DIG Easy Hyb	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. (To determine the T_m, add 4°C for each G or C and 2°C for each T or A. Perform prehybridization and hybridization 10°C below the calculated T_m). To prevent non-specific hybridization signals, hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly (A) in the prehybridization and hybridization solution. Additionally, 5 µg/ml of Poly (dA) may be added for further blocking.
tailed	0.1–2 pmol/ml		
end-labeled	1–10 pmol/ml	High SDS buffer	

*The conditions given here are stringent conditions, applicable if probe and target have 100% homology and a G plus C content of about 50%.

**When CDP-Star is used for detection, the recommended concentrations are 10–20 ng/ml DIG-labeled DNA or 20–50 ng/ml DIG-labeled RNA. Higher concentrations may cause background.

❷ Heat-denature the probe in a boiling water bath for 10 min.
Oligonucleotide probes do not require denaturation prior to dilution unless extensive secondary structure is predicted from the sequence.

❸ Dilute the probe in prehybridization solution. See Table 9 for recommended probe concentrations.

❹ Discard the prehybridization solution from the bag, and add the hybridization solution containing the DIG-labeled probe. Allow the probe to hybridize. See Table 9 for recommended hybridization conditions.

❺ At the end of the hybridization, pour the hybridization solution from the bag into a tube (with cap) that can withstand freezing and boiling (e.g., a 50 ml polypropylene tube).
This used hybridization solution contains unannealed DIG-labeled probe. The entire solution can be reused in future hybridization experiments. Store DIG-labeled DNA probes at –20°C; store DIG-labeled RNA probes at –70°C. DIG-labeled probes stored in this manner are stable for at least one year. For reuse, thaw and denature by heating to +68°C for 10 min.

❻ Wash the membrane twice, 15 min per wash, in 2 x wash solution at room temperature. These washes (steps 6 and 7) remove unbound probe, which would otherwise lead to high background.

❼ Wash the membrane twice, 15 min per wash, in 0.5 x wash solution. Wash long probes (> 100 bp) at +68°C. For shorter probes, the washing temperature must be determined empirically.

Note: The stringency of this final wash must be determined empirically. Depending on length and homology of the probe it will be necessary to adjust the salt concentration. Fully homologous probes will often require 0.1 x wash solution.

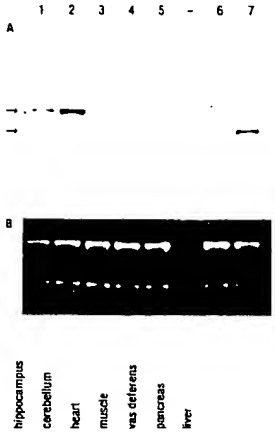


Figure 11: Example of a northern blot with a DIG-labeled RNA probe. Approximately 200 ng of total RNA from rat spinal cord (1), cortex (2), spleen (3), kidney (4), and liver (5, 6, 7) were run on a 1.5% agarose/formaldehyde gel and transferred to a nylon membrane. Specific mRNA was detected with a 2.5 kb digoxigenin-labeled antisense RNA probe derived from zinc finger cDNA. For quantification, lanes 6 and 7 contain 0.1 pg and 1 pg, respectively, of a synthetic sense RNA derived from the same cDNA.

A. 45 min exposure of the membrane 2 h after the start of the detection reaction with the chemiluminescence substrate. A 4.8 kb mRNA is detected in all tissues. The amount of mRNA in liver approximately corresponds to the 0.1 standard in lane 6 running at 2.5 kb. Arrows indicate the positions of the 18S and 28S ribosomal RNAs.

B. Photograph of the Ethidium bromide-stained 18S and 28S RNAs after transfer to a nylon membrane.

Data were kindly provided by U. Pott, Brain Research Institute, Zurich, Switzerland.

Figure 13: Extraction of mRNa from various rat tissues. mRNA was extracted from the indicated rat tissues (50 mg wet weight each) by the method described in reference 2. In order to determine the extraction efficiency, 8 pg of a shortened polyadenylated NGF recovery standard (reference 3) was added to each sample prior to mRNA extraction. Hybridization and detection were performed as described in Figure 12.
Data from Figures 12 and 13 were kindly provided by Dr. B. Hengerer, CIBA GEIGY AG, Basel, Switzerland.

Figure 12: Comparison of extraction efficiency. RNA was extracted from different amounts of mouse heart tissue after the addition of 4 pg recovery standard.
Lane 1: Total RNA was extracted from 80 mg heart tissue by the acid guanidinium thiocyanate-phenol-chloroform method as described in reference 1. **Lanes 2 to 6:** mRNA was extracted as described in reference 2 from 80 mg (lane 2), 16 mg (lane 3), 8 mg (lane 4), 1.6 mg (lane 5), and 0.8 mg (lane 6) heart tissue. 0.8 mg of heart tissue contains only about 50 ng Poly(A)$^+$ RNA and less than 300 fg nerve growth factor (NGF) mRNA, which is below the detection limit of conventional northern blots. The RNA was glyoxylated, separated in 1.2% agarose gel, and transferred to a positively charged Nylon Membrane (Boehringer Mannheim). After hybridization with a digoxigenin-labeled cRNA probe, NGF mRNA was visualized by chemiluminescent detection. Hybridization and detection were performed under standard conditions described in DIG-labeling and detection protocols from Boehringer Mannheim.

References

1. Chomczynski, P. and Sacchi, N. (1987) Single-step method for RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156.
2. Hengerer, B. (1993) A rapid procedure for mRNA extraction from a large number of samples. *BioTechniques* 14(4), 522–524.
3. Heuman, R. and Thoenen, H. (1986) Comparison between the time course of changes in nerve growth factor NGF protein levels and those of its messenger RNA, in the cultured rat iris. *J. Biol. Chem.* 261, 9246.

What to do next

Proceed to the "Detection" division of this User's Guide, which begins on page 58.

RNA Dot Blotting

Dot blots and slot blots are rapid methods for the qualitative screening of RNA. The same hybridization and detection procedures used with Northern blots are also performed on RNA dot blots; therefore, proceed to the Northern blotting application (page 53) after completing this dot blotting procedure.

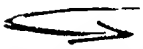
Required solutions

Solutions required for the hybridization and detection of RNA dot blots, but not specifically required by the dot blotting procedure given here, are listed in the Northern blotting application (page 53).

Required solution	Description
DMPC-treated H_2O	Sterile, distilled water, DMPC-treated with 0.1% dimethylpyrocarbonate (see page 84)
RNA dilution buffer	Mix DMPC-treated H_2O : 20 x SSC : Formaldehyde (5 : 3 : 2)

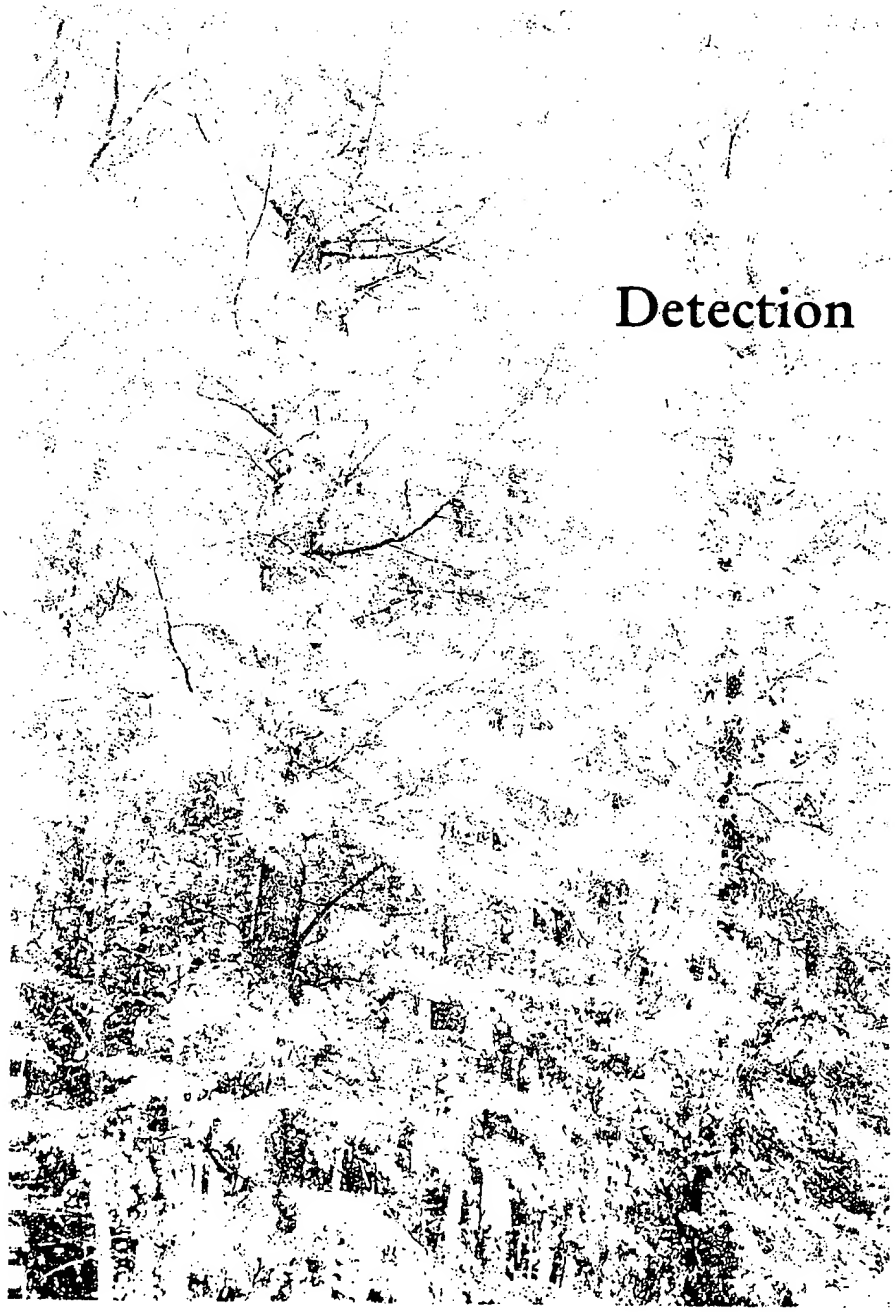
Procedure

1. Dilute the RNA sample in RNA dilution buffer.
2. Mark the membrane lightly with a pencil to identify each dilution before spotting.
3. Using a micropipettor, spot 1 µl of the RNA sample onto a dry nylon membrane. Alternatively, the sample can be applied using a slot- or dot-blotting manifold.
4. Fix the RNA to the membrane by UV crosslinking or baking in an oven at +120°C for 30 min. With nitrocellulose membranes, use a vacuum oven at +80°C for 2 h.



What to do next

Hybridize the samples on the dot blot according to the recommendations described in the northern blotting application, which begins on page 53.



Detection

Chemiluminescent Detection

Using chemiluminescent detection a light signal is produced on the site of the hybridized probe. The light signal can be recorded on X-ray films, requiring only very short exposure times.

Chemiluminescent detection is a three-step process. In the first step, membranes are treated with Blocking Reagent to prevent nonspecific attraction of antibody to the membrane. Then, membranes are incubated with a dilution of Anti-Digoxigenin, Fab fragments, which are conjugated to alkaline phosphatase. In the third step, the membrane carrying the hybridized probe and bound antibody conjugate is reacted with a chemiluminescent substrate and exposed to X-ray film to record the chemiluminescent signal.

Products required

Products and solutions required for chemiluminescent detection are listed below.

Chemiluminescent alkaline phosphatase substrates are available as:

- The DIG Luminescent Detection Kit (Cat. No. 1363514) contains all of the reagents required for chemiluminescent detection of digoxigenin-labeled nucleic acids, including CSPD®. It also contains a DIG-labeled control DNA for practicing chemiluminescent detection.
- CSPD® can be purchased as a separate reagent and used to replace the colorimetric detection reagents (BCIP and NBT) in the DIG DNA Labeling and Detection Kit (Cat. No. 1093657) or the DIG Nucleic Acid Detection Kit (Cat. No. 1175041).
- CPD-Star™ can be purchased as a separate reagent and used to replace the colorimetric detection reagents (BCIP and NBT) in the DIG DNA Labeling and Detection Kit (Cat. No. 1093657) or the DIG Nucleic Acid Detection Kit (Cat. No. 1175041).
- CSPD® and CDP-Star™ can both be used for the same applications. The choice for CSPD® or CDP-Star™ depends on your experience in working with chemiluminescent detection. CDP-Star™ is the fastest chemiluminescent substrate available, typically requiring exposure times of only 15–60 s, whereas exposure times for CSPD® are typically 15–30 min. Because working with CDP-Star™ requires some experience, we recommend to start your experiments with CSPD®.

Name in procedure	Description	Available as
Anti-Digoxigenin-AP*	750 units/ml Anti-Digoxigenin, Fab fragments conjugated to alkaline phosphatase	• Vial 3, DIG Luminescent Detection Kit for Nucleic Acids • Vial 8, DIG DNA Labeling and Detection Kit • Vial 3, DIG Nucleic Acid Detection Kit • Anti-Digoxigenin-AP (Cat. No. 1093274)
CSPD®	25 mM Disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl) phenyl phosphate (dilute before use)	• Vial 5, DIG Luminescent Detection Kit • CSPD® (Cat. No. 1655884)
CDP-Star™	25 mM disodium 4-chloro-3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl) phenyl phosphate (dilute before use)	• CDP-Star™ (Cat. No. 1685627)

Note: Small antibody aggregates in the Anti-Digoxigenin-AP may lead to background in the detection. It is therefore recommended to centrifuge the vial with antibody conjugate for 5 min at 13,000 rpm, before the first use. After the first use it is sufficient to centrifuge the Anti-Digoxigenin-AP for 1 min, directly before dilution.

Additionally required solutions

Except TE buffer, all the following required solutions are available in a ready-to-use form in the DIG Wash and Block Buffer Set (Cat. No. 1585762). Bottle numbers for this set are given in parentheses. Alternatively, they can be prepared from separate reagents according to procedures described in Appendix B. ▼

Additionally required reagent	Description
Washing buffer (Bottle 1; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 0.3% (v/v) Tween® 20
Maleic acid buffer (Bottle 2; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking solution (Bottle 3; dilute 1:10 with 1 x Maleic acid buffer)	1% (w/v) Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4°C, but must then be brought to room temperature before use.
Detection buffer (Bottle 4; dilute 1:10 with H_2O)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (+20°C)
TE buffer	10 mM Tris, 1 mM EDTA; pH 8.0 (+20°C)
DMPC-treated H_2O (for RNA probes only)	Sterile, distilled water treated with 0.1% dimethylpyrocarbonate (see page 84)

Guidelines for handling CSPD® or CDP-Star™

To maintain full activity, as well as the nuclease-, phosphatase-, and bacteria-free environment in which the chemiluminescent substrates CSPD® or CPD-Star™ are provided, adhere to the following precautions:

- ▶ Do not place any non-sterile instrument (e.g., pipet tips) into the substrate solutions.
- ▶ Remove the substrate from the bottle by pouring it into a sterile container using sterile technique or by transferring it with sterile pipettes. Wear powder-free gloves, and avoid touching the mouth of the bottle to anything.
- ▶ Avoid touching the membrane with fingers (gloved or ungloved).
- ▶ Use blunt-ended forceps that have been washed and autoclaved (to avoid alkaline phosphatase contamination) to pick up membranes, and handle membranes only at their edges.
- ▶ Wear unpowdered gloves, and use hybridization bags free of dust and powder. Gloves or bags can be washed in distilled water before use.
- ▶ Diluted CSPD® or CPD-Star™ can be stored at +4°C in the dark and can be reused one to two times.

Procedure

Perform all incubations at room temperature.

Incubations can be performed in a sealed hybridization bag or clean plastic tray. If a bag is used, remove all large air bubbles that may be present in the bag. If a tray is used, agitate the tray gently to ensure that the membrane is always covered. If you are using more than one membrane, add enough solution to cover all membranes.

❶ After hybridization and post-hybridization washes, equilibrate the membrane in washing buffer for 1 min.

❷ Allow the chemiluminescent substrate to come to room temperature.

❸ Using a freshly washed dish or bag, block the membrane by gently agitating it in blocking solution for 30–60 min. Near the end of the blocking period, prepare the antibody solution as described in step 4. Longer blocking times are acceptable.

❹ Dilute the Anti-Digoxigenin-AP 1:10,000 (after centrifugation, see page 58) in blocking solution. Mix gently by inversion. For example, for a 1:10,000 dilution, add 3 µl Anti-Digoxigenin-AP to 30 ml blocking solution and mix. When working with CDP-Star™, dilute Anti-Digoxigenin-AP 1:20,000 in blocking solution.
This working antibody solution is stable for about 12 h at +4°C.

❺ Pour off the blocking solution and incubate the membrane for 30 min in the antibody solution prepared in step 4.

❻ Discard the antibody solution. Gently wash the membrane twice, 15 min per wash, in washing buffer.

❼ Pour off washing buffer and equilibrate the membrane in detection buffer for two min. Dilute CSPD® or CDP-Star™ 1:100 in detection buffer. It is important that the filter is kept wet before the chemiluminescent substrate is applied. If the membrane is even slightly dry, high background can occur.

❽ There are two methods of applying the diluted substrate. The single-filter method should be used when DNA is to be visualized on a single membrane. The filter-batching method is recommended for multiple membranes but may also be used when visualization is performed on a single membrane.

Single-filter method

a. Place the membrane between two sheets of acetate (plastic page protectors). Gently lift the top sheet of plastic and, with a sterile pipet, add approximately 0.5 ml (per 100 cm²) of the chemiluminescent substrate on top of the membrane, scattering the drops over the surface of the membrane. Lower the top sheet of plastic. With a damp lab tissue gently wipe the top sheet to remove any bubbles present under the sheet and to create a liquid seal around the membrane. Incubate filter for 5 min. Proceed to step 9.

Filter-batching method

a. Pipette 5–10 ml of diluted CSPD® or CDP-Star™ into the center of sterile dish. Using blunt-end forceps, place the membrane in the dish. Tilt the dish until the membrane is thoroughly saturated.
b. Incubate filter for 5 min. Remove the membrane from the substrate, and allow any excess liquid to drip off. Do not allow the membrane to dry.
c. Cover the damp membrane by placing it between two clear acetate sheets or page protectors.
d. Wipe the top sheet with a damp lab tissue to remove any bubbles present between the sheet and the membrane.
e. Repeat the filter-batching method until the chemiluminescent substrate has been applied to all membranes. To prevent the membrane from drying out, avoid repeated exposure to air. After treating the final membrane, proceed to step 9.

❾ Seal the semi-dry membranes in a plastic bag.

❿ For the briefest exposure to X-ray film, the alkaline phosphatase chemiluminescent reaction must be at a steady state. At room temperature, 7–8 h are required to reach a steady state reaction. Once a steady state is reached, single-copy gene detection on a human genomic blot can be obtained with an approximate exposure time of 15 min. If the membrane is exposed before the steady state is reached, approximately 60 min of exposure is required for single-copy gene detection on a human genomic blot. Therefore, to shorten exposure times, we recommend incubation of the membrane for 15 min at +37°C before exposure to X-ray film.
Note: Because CDP-Star™ shows higher initial signals, this incubation step is neither necessary, nor recommended when CDP-Star™ is used.

⓫ For detection of the chemiluminescent signal, the membrane is exposed to Lumi-Film (Boehringer Mannheim) or standard X-ray film. Multiple exposures from a single blot can be obtained for up to 2 days after the addition of the chemiluminescent substrate. As starting point for the exposure time we recommend 15–20 min (CSPD®) or 1 min (CDP-Star™). Adjust the exposure time to the signal strength.

Colorimetric Detection with NBT and BCIP

With the DIG System, detection can be performed with the colorimetric detection reagents NBT and BCIP (X-Phosphate).

Products required

Products and solutions required for colorimetric detection are listed below. The colorimetric reagents are available separately, in the DIG DNA Labeling and Detection Kit (Cat. No. 1093 657), or in the DIG Nucleic Acid Detection Kit (Cat. No. 1175 041). ▼

Name in procedure	Description	Available as
Anti-Digoxigenin-AP*	750 units/ml Anti-Digoxigenin, Fab fragments conjugated to alkaline phosphatase	• Vial 8, DIG DNA Labeling and Detection Kit • Vial 3, DIG Nucleic Acid Detection Kit • Anti-Digoxigenin-AP, Fab fragments (Cat. No. 1093 274)
NBT solution	75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide	• Vial 9, DIG DNA Labeling and Detection Kit • Vial 4, DIG Nucleic Acid Detection Kit • NBT [Cat. No. 1383 213 (sold as 100 mg/ml; dilute prior to use)]
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in 100% dimethylformamide	• Vial 9, DIG DNA Labeling and Detection Kit • Vial 4, DIG Nucleic Acid Detection Kit • BCIP (Cat. No. 1383 221)

Additionally required solutions

Except TE buffer, all of the following required solutions are available in a ready-to-use form in the DIG Wash and Block Buffer Set (Cat. No. 1585762). Bottle numbers for this set are indicated in parentheses. Alternatively, they can be prepared from separate reagents according to procedures described in Appendix B. ▼

Additionally required reagent	Description
Washing buffer (Bottle 1; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 0.3% (v/v) Tween® 20
Maleic acid buffer (Bottle 2; dilute 1:10 with H_2O)	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking solution (Bottle 3; dilute 1:10 with 1 x Maleic acid buffer)	1% (w/v) Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer. Blocking solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at +4°C, but must then be brought to room temperature before use.
Detection buffer (Bottle 4; dilute 1:10 with H_2O)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (+20°C)
TE buffer	10 mM Tris, 1 mM EDTA; pH 8.0 (+20°C)
DMPC-treated H_2O (for RNA probes only)	Sterile, distilled water treated with 0.1% dimethylpyrocarbonate (see page 84)

Procedure

Perform all incubations at room temperature.

1. After hybridization and post-hybridization washes, equilibrate the membrane in washing buffer for 1 min.
2. Using a freshly washed dish or bag, block the membrane by gently agitating it in blocking solution for 30–60 min. Near the end of the blocking period, prepare the antibody solution as described in step 3.
   Longer blocking times are also acceptable.

For sufficient blocking, there must be ample room in the bag or dish to allow for unrestricted shaking of the membrane. If you are using more than one membrane, add enough solution to cover all membranes.

**Note: Small antibody aggregates in the Anti-Digoxigenin-AP may lead to background in the detection. It is therefore recommended to centrifuge the vial with antibody conjugate for 5 min at 13,000 rpm, before the first use. After the first use it is sufficient to centrifuge the Anti-Digoxigenin-AP for 1 min, directly before dilution.*

❺ Dilute the Anti-Digoxigenin-AP 1:5,000 (after centrifugation) in blocking buffer for a working concentration of 150 mU/ml. Mix gently by inversion. For example, add 6 µl Anti-Digoxigenin-AP to 30 ml blocking solution, and mix.
This working antibody solution is stable for about 12 h at +4°C.

❻ Pour off the blocking solution, and incubate the membrane for 30 min in the antibody solution prepared in step 3. If a bag is used, remove all large air bubbles that may be present in the bag. If a tray is used, agitate the tray gently to ensure that the membrane is always covered.

❼ Discard the antibody solution. Wash twice, 15 min per wash, in 100 ml washing buffer. These washes remove unbound antibody.

❽ Mix 45 µl NBT solution and 35 µl BCIP solution in 10 ml of detection buffer. This freshly prepared color substrate solution will be used in step 8. Protect from direct light before use.

❾ Equilibrate the membrane in 20 ml detection buffer for 2 min.

❿ Pour off the detection buffer, and add approximately 10 ml color substrate solution to the membrane. Incubate the membrane in a sealed plastic bag or box in the dark. Do not shake the container while the color is developing.
The membrane can be exposed to light for short periods to monitor the color development. The color precipitate starts to form within a few min, and the reaction is usually complete after 12 h. Do not shake.

⓫ Once the desired spots or bands are detected, wash the membrane with H_2O to prevent over-development. If the membrane is to be reused, use sterile H_2O or a sterile buffer (e.g., TE buffer) to stop the development.

Results can be documented by photocopying the wet filter or by photography. Photocopying onto overhead transparencies allows for densitometric scanning; to do this, the color reaction can be interrupted for a short time and continued afterwards.

The membrane can also be dried at room temperature, and then stored, although the color fades upon drying. To revitalize the color, wet the membrane in TE buffer. If the membrane is to be reprobed, do not allow the membrane to dry.

Alternatively, store the membrane in a sealed plastic bag containing TE buffer. In this case, the color remains unchanged.

Multicolor Detection

Detection of digoxigenin-, biotin-, and fluorescein-labeled nucleic acids can be performed with successive enzyme immunoassays that yield three different colors.

The multiple-labeling and multicolor detection scheme allows discrete nucleic acid sequences to be detected with differently colored hybridization signals on the same blot. Nucleic acid probes labeled with digoxigenin, fluorescein, or biotin are hybridized simultaneously to immobilized target nucleic acids. The labels are detected by alkaline phosphatase conjugates (Anti-Digoxigenin-alkaline phosphatase, Anti-Fluorescein-alkaline phosphatase, or Streptavidin-alkaline phosphatase) and three different naphthol-AS-phosphate/diazonium salt combinations as substrates for alkaline phosphatase. The detection reactions are carried out consecutively, with a heat/EDTA treatment between each to inactivate the formerly bound alkaline phosphatase. The resulting hybridization signals are green, red, or blue for targets that have homology to only one probe. If the target DNA or RNA fragment is homologous to more than one of the probes, the resulting signal is a mixed color.

This method is of advantage in all applications where different hybrids are to be detected on the same blot or specimen. Useful applications include genomic Southern blots of lower eukaryotes, plasmid mapping, northern blots comparing the abundance of different mRNAs, and colony and plaque hybridizations.

Multicolor detection allows 0.3 pg of homologous DNA to be detected within 2 h; this sensitivity is satisfactory for single-copy gene detection in genomic blots of lower eukaryotes like yeast or *Drosophila*, but can sometimes be insufficient for genomic blots of mammalian DNA. When greater sensitivity is required, use the colorimetric BCIP/NBT substrate or the chemiluminescent substrates CSPD® or CDP-Star™.

Products required

Products and solutions required for multicolor detection are listed below. Refer to Appendix B for details on preparing the additionally required solutions. ▼

Name in procedure	Description	Available as
Anti-Digoxigenin-AP	Anti-Digoxigenin, Fab fragments conjugated to alkaline phosphatase	• Vial 8, DIG DNA Labeling and Detection Kit • Vial 3, DIG Nucleic Acid Detection Kit • Anti-Digoxigenin-AP, Fab fragments (Cat. No. 1093 274)
Anti-Fluorescein-AP	Anti-Fluorescein, Fab fragments conjugated to alkaline phosphatase	• Anti-Fluorescein-AP, Fab fragments (Cat. No. 1426 388)
Streptavidin-AP	Streptavidin conjugated to alkaline phosphatase	• Streptavidin-AP (Cat. No. 1093 266)
Multicolor Detection Set	Alkaline phosphatase substrate tablets for 3 x 50 detection reactions (for the detection of 60 blots of 10 x 10 cm² with three colors) The set contains: • "Green" AP substrate tablets; 50 tablets, each containing 2 mg of naphthol-AS-Gr-phosphate and 3.5 mg of Fast Blue B • "Red" AP substrate tablets; 50 tablets, each containing 2 mg of naphthol-AS-phosphate and 1 mg of Fast Red TR • "Blue" AP substrate tablets; 50 tablets, each containing 2 mg of naphthol-AS-phosphate and 3.5 mg of Fast Blue B	• Multicolor Detection Set (Cat. No. 1 465 341)
Blocking Reagent	Blocking reagent for nucleic acid hybridization	• Blocking Reagent (Cat. No. 1096176)

Additionally required solutions

In addition, you will need the following solutions. See Appendix B for solution preparation.

Additionally required reagent	Description
EDTA	50 mM EDTA, pH 8.0
Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl; pH 7.5 (+20°C); adjusted with solid or concentrate NaOH, autoclaved
Washing buffer	100 mM maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 3% (v/v) Tween® 20
TE buffer	10 mM Tris, 1 mM EDTA; pH 8.0 (+20°C)
Blocking reagent stock solution	Blocking reagent for nucleic acid hybridization dissolved in Maleic acid buffer to a final concentration of 10% (w/v); afterwards the solution is autoclaved and stored at +4°C or –20°C
Blocking solution for DIG- and fluorescein-labeled probes	Sterile Blocking reagent stock solution diluted 1:10 in Maleic acid buffer (final concentration = 1% Blocking Reagent)
Blocking solution for biotin-labeled probes	Sterile Blocking reagent stock solution diluted 1:2 in Maleic acid buffer (final concentration = 5% Blocking Reagent)
Antibody solutions	Anti-Digoxigenin-alkaline phosphatase, Anti-Fluorescein-alkaline phosphatase or Streptavidin-alkaline phosphatase, each 150 mU/ml in the appropriate blocking solution
Detection buffer	0.1 M Tris-HCl, pH 9.5 (+20°C); 0.1 M NaCl
Color substrate working solutions (freshly dissolve in Detection buffer)	Prepare fresh working solution each day. Dissolve one tablet in 10 ml Detection buffer while stirring at room temperature. The "Red" and "Blue" tablets dissolve completely within a few minutes. The "Green" tablets do not dissolve completely, but this does not influence the results. Note: We recommend that gloves be worn when handling the color substrate solutions.

Labeling with Fluorescein or Biotin

As with DIG labeling, nucleic acid probes can be labeled with fluorescein or biotin by random-primed DNA labeling, DNA amplification by PCR, *in vitro* RNA transcription, or oligonucleotide end-labeling or tailing. The labels can also be introduced into oligonucleotides via chemical synthesis. Fluorescein- and biotin-labeled probes are most conveniently prepared with the High Prime Fluorescein DNA Labeling Mix (Cat. No. 1585622) and High Prime Biotin DNA Labeling Mix (Cat. No. 1585649). Fluorescein-12-dUTP and Biotin-16-dUTP are also available as single reagents, and can replace DIG-11-dUTP in the DIG Kit protocols. Labeling protocols for these nucleotide analogs are given in the respective pack inserts. The labeling reactions can also be carried out without kits using single reagents; a listing of the single reagents for nonradioactive labeling is given in Appendix C.

Hybridization

The differently labeled probes can be hybridized simultaneously to a blot or *in situ* to homologous sequences according to the protocols given for hybridization of DIG-labeled probes.

It is especially important that the probe concentration for hybridization be optimized for all three probes. We strongly recommend a mock hybridization on a naked piece of membrane or a dot blot for this evaluation. A protocol for this is given on page 42.

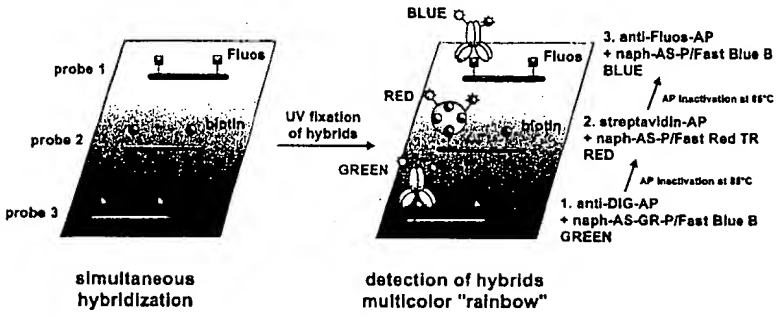
If multiple (multicolor) detections are performed on the same membrane, the hybrids must be fixed to the membrane after the stringency washes (but before detection). This is necessary because heat treatment is used to inactivate the alkaline phosphatase between detections. To crosslink the hybrids, expose the membrane to UV light for 3 min at 254 nm. If only one label and one color has been used, fixation of the hybrids is not necessary.

Multicolor Detection

Detection of the different labels is performed by binding the respective antibody- or streptavidin-alkaline phosphatase conjugate. A different combination of naphthol-phosphate and diazonium salt is used to yield a different color for each conjugate (green, blue, or red).

The detection reactions are performed consecutively, with heat-inactivation of the alkaline phosphatase between the detections. As stated above, hybrids must be stabilized by UV-crosslinking (3 min at 254 nm) if different labels are detected consecutively.

Figure 14: Principle of Multicolor Detection Set.



The following procedure describes triple detection; orders of detection and colors may be changed according to individual requirements.

All incubations are performed at room temperature and, except for the color reaction, with shaking or mixing. The volumes of the solutions are calculated for a membrane size of 100 cm^2, and should be adjusted to fit other membrane sizes. Blocking and equilibration steps may proceed for longer periods if more convenient.

Detection of DIG- or Fluorescein-labeled Hybrids

Procedure

1. If multicolor detection is to be performed, fix hybrids by UV exposure (3 min at 254 nm) or baking. This should be performed after hybridization and stringency washes.
2. Wash the membrane briefly in washing buffer.
3. Incubate for at least 30 min with about 100 ml of blocking solution for DIG- and fluorescein-labeled probes (1% [w/v]).
4. Dilute the Anti-Digoxigenin-alkaline phosphatase or Anti-Fluorescein-alkaline phosphatase 1:5,000 in blocking solution for DIG- and fluorescein-labeled probes (final concentration, 150 mU/ml).
   These diluted antibody-conjugate solutions are stable for about 12 h at +4°C.
5. Incubate the membrane for 30 min in about 20 ml of the diluted antibody conjugate solution prepared in step 4.
6. Wash twice, 15 min per wash, with 100 ml of washing buffer.
   These washes remove unbound antibody conjugate.
7. Equilibrate the membrane for at least 2 min in 20 ml detection buffer.
8. Dissolve one substrate tablet in 10 ml detection buffer. Incubate the membrane with 10 ml of one of the color substrate solutions (freshly prepared) for about 45 min, then replace with fresh color substrate solution if necessary.
   The colored precipitate begins to form within a few minutes and can be allowed to proceed for up to 2 h (until the desired signal intensity is obtained).
9. Inactivate the alkaline phosphatase as described below or stop the final color reaction by washing the membrane with TE buffer.

Alkaline Phosphatase Inactivation

(to be performed between any two detections)

1. Wash the membrane briefly in TE buffer.
2. Incubate the membrane for at least 10 min at +85°C in EDTA.
3. Wash the membrane twice for 5 min in Washing buffer.
   This wash removes the EDTA.
4. Proceed to the next detection procedure.

Detection of Biotin-labeled Hybrids

1. If multicolor detection is to be performed, fix hybrids by UV exposure (3 min at 254 nm) or baking. This should be performed after hybridization and stringency washes.
2. Wash the membrane briefly in washing buffer.
3. Incubate the membrane for at least 30 min in about 100 ml blocking solution for biotin-labeled probes (5% [w/v]).
4. Dilute Streptavidin-AP 1:5000 in blocking solution for biotin-labeled probes (final concentration, 150 mU/ml).
5. Incubate the membrane for 30 min in about 20 ml diluted streptavidin-conjugate solution prepared in step 4. Diluted streptavidin-conjugate solutions are stable for about 12 h at +4°C.
6. Wash twice, for 15 min per wash, in 100 ml of washing buffer.
   These washes remove unbound conjugate.
7. Equilibrate the membrane for at least 2 min in 20 ml detection buffer.
8. Dissolve one tablet in 10 ml detection buffer. Incubate the membrane with 10 ml freshly prepared color substrate solution for about 45 min, then replace with fresh color substrate solution if necessary.
   The colored precipitate begins to form within a few minutes and can be allowed to proceed for up to 2 h (until the desired signal intensity is obtained).
9. Inactivate the alkaline phosphatase as described above or stop the final color reaction by washing the membrane with TE buffer.

Results can be documented by photography. The colors do not fade when the membranes are dried and stored at room temperature.

Stripping and Reprobing of Membranes
Membranes can only be reused if only one label and color has been used and if the hybrids have not been UV-crosslinked or baked. Do not allow the membrane to dry out if it is to be reprobed.

Removal of color precipitate

The color precipitate can be removed by washing the membrane with ethanol (red: room temperature; blue and green: +50° to 65°C; put the membrane and ethanol in a sealed plastic bag in a hybridization oven or waterbath). Replace the solution from time to time until the precipitate is completely dissolves.

Removal of the probe

1. Thoroughly rinse the membrane in water.
2. Incubate twice for 20 min in 0.2 M NaOH, 0.1% (w/v) SDS at + 37°C.
3. Rinse the membrane in 2 x SSC. The membrane may now be dried or used directly for hybridization. Alternatively, any established procedure for removing hybridized probes (e.g., heating in SDS buffer or formamide-containing buffers at neutral pH) can be used.

Reference

1. Höltke, H. J., Ettl, I., Finken, M., West, S. and Kunz, W. (1992) "Multiple Nucleic Acid Labeling and Rainbow Detection." *Anal. Biochem.* 207, 24–31.

Stripping Membranes for Reprobing

Stripping of blots that have been detected colorimetrically is only possible when nylon membranes were used for blotting. The color precipitate has to be removed by incubation in dimethylformamide (DMF) and nitrocellulose is dissolved under such conditions.

The luminescent signal can be easily removed by a short wash of the filter in water.

For the subsequent removal of probe there are several procedures. With the procedures described below we have achieved good results.

Note: When a membrane is to be rehybridized the membrane should be kept wet at all stages between hybridization and probe removal

▼

Required solutions

Additionally required reagent	Description
Dimethylformamide	100% ACS grade N,N-dimethylformamide (DMF)
H_2O	Sterile, distilled water
Alkaline probe-stripping solution	0.2 NaOH, 0.1% SDS
2 x SSC buffer	300 mM NaCl, 30 mM sodium citrate
2 x SSC/0.1% SDS	300 mM NaCl, 30 mM sodium citrate, 0.1% (w/v) SDS
Northern probe-stripping solution	50% formamide; 50 mM Tris-HCl, pH 8; 1% (w/v) SDS
DMPC-treated H_2O	Sterile, distilled water treated with 0.1% dimethylpyrocarbonate (see page 84)

Procedure for removing the color precipitate

1. Using a water bath, heat a large glass beaker of dimethylformamide to +50–60°C.
   Caution: Dimethylformamide is volatile and flammable. Keep away from sparks and open flames. Work in a fume hood. The flash point of dimethylformamide is at +67°C.
2. Incubate the membranes in the heated dimethylformamide until the blue color has been removed.
   Changing the dimethylformamide solution frequently will increase the speed of decolorization.
3. Rinse the membranes thoroughly in H_2O.
4. Proceed to probe removal.
   Caution: Do not allow the membrane to dry prior to probe removal.

Procedure for removing the chemiluminescent substrate

1. Wash the membrane in H_2O for 1 min.
2. Proceed to probe removal.
   Caution: Do not allow the membrane to dry prior to probe removal.

Procedure for removing the probe from Southern, DNA dot, and colony/plaque hybridizations

1. Wash the membranes in H_2O for 1 min.
2. Incubate the membranes twice for 10 min Alkaline probe-stripping solution at +37°C. This incubation removes the alkali-labile DIG-labeled probe.
3. Rinse the membranes thoroughly in 2 x SSC.
4. Commence reprobing with the prehybridization step of the desired hybridization procedure.

Procedures for removing the probe from Northern blots

Note: Due to the stability of RNA-RNA hybrids, stripping of Northern blots is not always successful. We offer you here 2 methods that have proven to be successful in some cases. However, always take possible problems with stripping into consideration, when multiple hybridizations to the same RNA are intended.

Method I

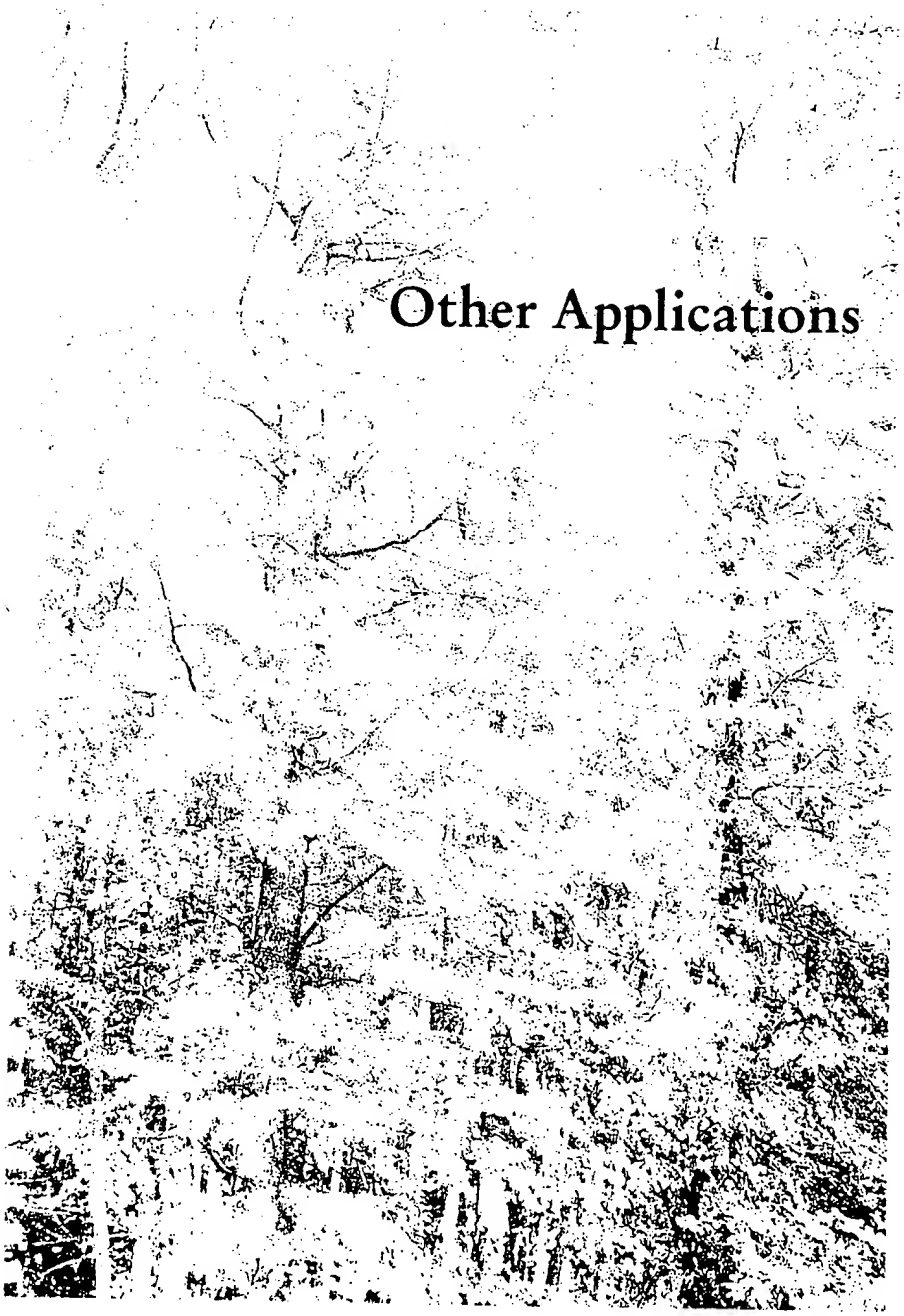
1. Heat 100 ml of 0.1% SDS in a 500 ml beaker.
2. Shortly before the SDS-solution starts to boil, transfer the membrane to a clean tray.
3. Pour the boiling SDS-solution over the membrane.
4. Incubate for 10 min on a rocking platform (i.e. without further heating).
5. Wash for 5 min in Washing buffer at room temperature.
6. Proceed to the prehybridization.

Method II

1. Rinse the membrane thoroughly in sterile H_2O.
2. Incubate the membrane twice, 30 min per incubation, in Northern probe-stripping solution at +68°C.
3. Rinse the membrane, first in water, then in 2 x SSC.
4. Commence reprobing with the prehybridization step of the desired hybridization procedure.

Note:

- After stripping, start with the prehybridization or store the filter wet in 2 x SSC in a sealed plastic bag.
- Prewarm the stripping solution to the appropriate incubation temperature.
- For incubation, use a shaking waterbath or a hybridization oven.



Other Applications

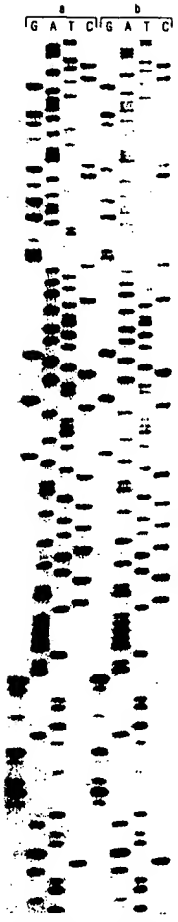


Figure 15: Partial sequence of pUC 18, obtained by DIG cycle sequencing of a PCR product, obtained with (a) unlabeled PCR primers and DIG-labeled sequencing primers of the same sequence or (b) DIG-labeled PCR primers and DIG-labeled sequencing primers of the same sequence.

Chapter 10 • Other Nonradioactive Molecular Biology Applications

Besides the in this User's Guide presented applications for filter hybridization, Boehringer Mannheim offers a growing range of reagents for nonradioactive analysis. Kits and reagents are offered for

- nonradioactive *in situ* hybridization
- reporter gene assays
- protein labeling and detection
- cell proliferation/cell death assays.

Here we present 3 applications that are directly related to the analysis of nucleic acids; sequencing, gel shift assays and *in vitro* translation. The individual kits come with a detailed protocol. For additional information please contact your local representative.

Nonradioactive Sequencing

Products offered for this application (see Appendix C, page 88 for a complete listing) ▼

Product	Cat. No.	Size
DIG Taq DNA Sequencing Kit for Standard and Cycle Sequencing**	1 449 443	1 kit (100 reactions)
Luminescent Detection Set for DIG Sequencing	1 733 915	1 set (10,000 cm^2 membrane)

The DIG system for sequencing double- and single-stranded DNA with Taq DNA Polymerase is based on the chain termination principle of Sanger.

Taq DNA Polymerase ir a highly processive 5'-3' DNA Polymerase that lacks 3'-5' exonuclease and shows highest activity at temperatures around 75°C. Because of these properties, Taq DNA Polymerase is ideally suited for DNA sequencing, and the thermostability of the enzyme allows cycle sequencing when only low amounts of sequencing template are available.

The primary advantage to sequencing at elevated temperatures is that premature termination sequences are avoided. These troublesome stops are the result of exceptional secondary structure, which is eliminated at higher temperatures. This, in combination with the use of 7-Deaza-dGTP, allows the sequencing and resolution of templates where other polymerases fail.

Principle

A sequencing primer*, 5'-end labeled with digoxigenin, is hybridized to the sequence template. The primer-template hybrid is divided over 4 tubes, each containing a termination mix for one of the the four different bases. In a one step reaction (standard sequencing) or in a cyclic reaction (cycle sequencing) the primer is elongated by Taq DNA Polymerase. Incorporation of a dideoxynucleotide leads to the termination of the elongation reaction.

The terminated sequencing products are then separated on a denaturing polyacrylamide gel. To enable visualization of the DIG-moieties, present at the 5'-end of every elongated strand, the products must now be transferred to a nylon membrane. This is most conveniently performed by a 20 min contact blot procedure or by vacuum blotting. Alternatively, the sequence products can be transferred directly to the membrane by Direct Blotting Electrophoresis (DBE), using an appropriate device.

Once the products have been transferred to a nylon membrane, detection is performed analogous to the procedures described in the "Detection"-section of this User's Guide. The membranes are generally very large and it is recommended to use lower concentrations of chemiluminescent substrate for economical reasons. This would normally lead to longer exposure times, but when using the Luminescent Detection Set for DIG Sequencing (with CDP-Star™ as substrate), visualization can still be accomplished within 15–30 min. A typical result is shown in figure 15.

*In the DIG Taq DNA Sequencing Kit for Standard and Cycle Sequencing a M13/pUC sequencing and a M13/pUC reverse sequencing primer, 5'-digoxigenin-labeled, are contained. Other primers are available (see appendix C, page 88) or can be synthesized by suppliers of custom-made oligonucleotides.

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Nonradioactive Gel Shift Assay

Product offered for this application

Product	Cat. No.	Size
DIG Gel Shift Kit	1635352	1 kit for 20 labeling reactions, 200 gel shift reactions and detection reagents for 20 blots. Includes control DNA binding protein (Oct2A) and control oligonucleotide.

The study of DNA-protein interactions has been significantly facilitated in recent years by the "gel retardation" or "gel mobility shift" assay. This rapid and simple technique is based on the separation of free DNA from DNA-protein complexes, due to the differences in their electrophoretic mobilities in native (non-denaturing) polyacrylamide or agarose gels.

Principle

DNA probes are 3'-end labeled with terminal transferase and DIG-ddUTP using the reagents provided in the DIG Gel Shift Kit. The labeled DNA fragment, containing the sequence of interest, is incubated with cell extract or (partly) purified DNA binding protein. The mixture is then transferred onto a native polyacrylamide gel and submitted to gel electrophoresis. The separated fragments can be transferred to a nylon membrane by electroblotting, capillary transfer or contact blotting.

Following blotting, the DIG-labeled DNA-fragments are detected by an enzyme immunoassay, described in detail in the "Detection" section of this Guide. Anti-Digoxigenin-alkaline phosphatase conjugate and the chemiluminescent substrate CSPD are provided in this kit.

A typical result is shown in Figure 16.

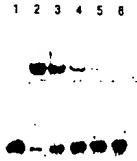


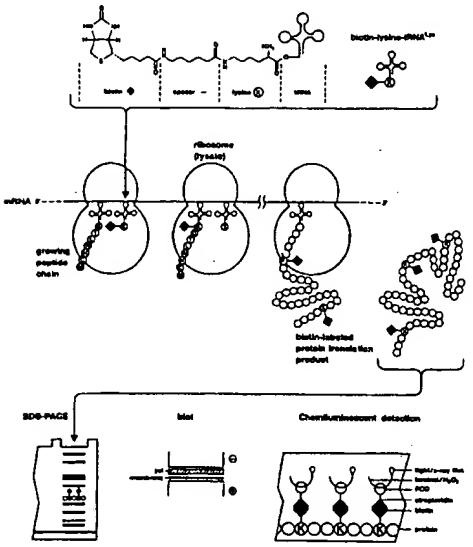
Figure 16: DIG Gel Shift Assay with purified Oct2A. The binding reactions were separated on a 12,5% native homogeneous polyacrylamide gel. Oligonucleotides were transferred onto a nylon membrane by electroblot. The membrane was exposed to X-ray film for 30 min at room temperature. Lane 1: DIG-labeled oligonucleotide (0.8 ng) containing Oct2A-binding site, Lane 2: DIG-labeled oligonucleotide (30 fmol) containing Oct2A-binding site, incubated with 50 ng of purified Oct2A, Lanes 3–6: DIG-labeled oligonucleotide (30 fmol) containing Oct2A-binding site, incubated with 50 ng of purified Oct2A, and with 25-, 62-, 125-, and 250-fold molar excess of unlabeled oligonucleotide, respectively.

Nonradioactive *In Vitro* Translation

Product offered for this application

Product	Cat. No.	Size
Biotin *In Vitro* Translation Kit	1 559 451	1 kit for 30 translation reactions

Figure 17: Principle of biotin *in vitro* translation and chemiluminescent detection. ▼



The incorporation of biotin into proteins during *in vitro* translation is a nonradioactive alternative to using radiolabeled amino acids. Following translation, biotin-labeled proteins can be visualized in a blot format. Biotin-labeled translation products can also be modified and processed with canine pancreatic microsomes.

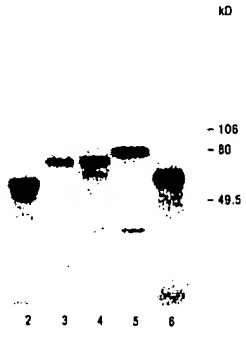
Principle

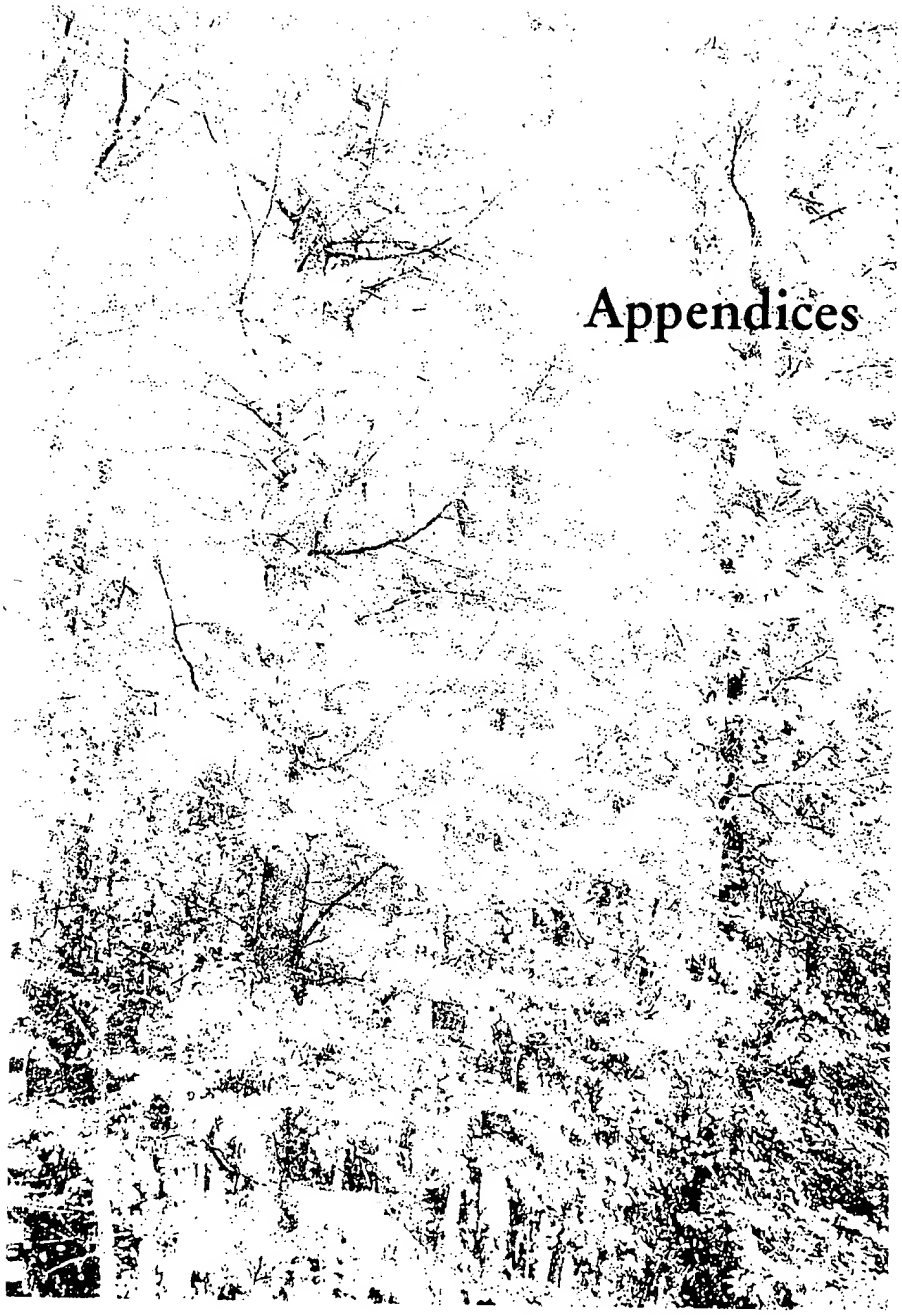
With the Biotin *In Vitro* Translation Kit, experimental RNA (cellular mRNA, viral RNA or *in vitro* transcribed RNA can be used) is translated into a biotin labeled protein, using a reticulocyte lysate. This lysate contains a charged lysine-tRNA, which is labeled with biotin at the ε-amino group of lysine (see Figure 17). All the required components for nonradioactive *in vitro* translation are provided in the kit in a premixed and ready-to-use form (1 tube/1 reaction); only the RNA of interest, water, and in some cases additional salts have to be added.

Aliquots of the biotin-labeled translation products are electrophoretically separated on a SDS-polyacrylamide gel and electroblotted onto a PVDF or nitrocellulose membrane. The biotin-labeled reaction products are subsequently detected with the BM Chemiluminescence Western Blotting Kit (biotin/streptavidin), (Cat. No. 1 559 460), using a streptavidin-horseradish peroxidase conjugate and luminol/iodphenol as substrate. The chemiluminescent signals are recorded by exposure to X-ray film for a few seconds up to 10 min.

A typical result is shown in Figure 18.

Figure 18: Biotin *in vitro* translation. ▶ RNA, coding for different proteins were translated with the Biotin *in vitro* Translation Kit. 2 µl per reaction were separated by SDS-PAGE and blotted onto a PVDF membrane. The detection with Streptavidin-POD and luminol/iodphenol was recorded by exposure to X-ray film for 1 min.
Lane 1: Control without RNA,
Lane 2: γ-globuline, Lane 3: Tissue Plasminogen Activator (tPA),
Lane 4: Transcription Factor CTF1,
Lane 5: Luciferase, Lane 6: Factor IX.





Appendices

Appendix A • DIG Kits' Contents

DIG High Prime Labeling and Detection Starter Kit I (for color detection with NBT/BCIP) 1745832

Vial	Description	Function	Cat. No. (if available separately)
1	one vial containing 50 µl DIG-High Prime; 5 x conc. labeling mixture containing optimal concentrations of random primers, nucleotides, DIG-dUTP (alkali-labile), Klenow enzyme and buffer components	used for the highly efficient random primed labeling of DNA	1585606
2	one vial containing 20 µl Unlabeled Control DNA (200 µg/ml pBR328 DNA that has been linearized with *Bam* HI)	DNA template for control reaction	
3	one vial containing 1 ml DNA dilution buffer (50 µg/ml herring sperm DNA in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+ 25°C])	used to make dilutions of the labeled DNA for the estimation of the labeling efficiency	
4	one vial containing 100 µl Anti-Digoxigenin-AP conjugate (750 U/ml polyclonal sheep anti-digoxigenin, Fab-fragments, conjugated to alkaline phosphatase)	binds to incorporated digoxigenin	1093274
5	six vials, each containing 1 ml NBT/BCIP 50 x conc. stock solution (18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 67 % (v/v) dimethyl formamide)	precipitating chromogenic substrate for alkaline phosphatase	1681451
6	4 bottles, each containing 100 ml Blocking Reagent 10 x conc.	blocks non-specific binding of probe and/or antibody to the membrane	1096176 (dry powder)
7	one vial containing 5 DIG Quantification Test Strips	used for the quantification of the yield of probe labeling	1669958
8	one vial containing 5 DIG Control Test Strips	used as control for the quantification of the yield of probe labeling	1669966

DIG High Prime Labeling and Detection Starter Kit II (for chemiluminescent detection with CSPD®, ready to use) 1 585 614

Vial	Description	Function	Cat. No. (if available separately)
1	one vial containing 50 µl DIG-High Prime; 5 x conc. labeling mixture containing optimal concentrations of random primers, nucleotides, DIG-dUTP (alkali-labile), Klenow enzyme and buffer components	used for the highly efficient random primed labeling of DNA	1 585 606
2	one vial containing 20 µl Unlabeled Control DNA (200 µg/ml pBR328 DNA that has been linearized with *Bam* HI)	DNA template for control reaction	
3	one vial containing 1 ml DNA dilution buffer (50 µg/ml herring sperm DNA in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 (+ 25°C))	used to make dilutions of the labeled DNA for the estimation of the labeling efficiency	
4	one vial containing 100 µl Anti-Digoxigenin-AP conjugate (750 U/ml polyclonal sheep anti-digoxigenin, Fab-fragments, conjugated to alkaline phosphatase)	binds to incorporated digoxigenin	1 093 274
5	one dropper bottle containing 50 ml CSPD®, ready-to-use (0.25 mM disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}] decan)-4-yl) phenyl phosphate)	chemiluminescent substrate for alkaline phosphatase	1 755 633
6	4 bottles, each containing 100 ml Blocking Reagent 10 x conc.	blocks non-specific binding of probe and/or antibody to the membrane	1 096 176 (dry powder)
7	one vial containing 8 DIG Quantification Test Strips	used for the quantification of the yield of probe labeling	1 669 958
8	one vial containing 5 DIG Control Test Strips	used as control for the quantification of the yield of probe labeling	1 669 956
9	one vial containing 1 ml NBT/BCIP 50 x conc. stock solution (18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 67 % (v/v) dimethyl formamide)	precipitating chromogenic substrate for alkaline phosphatase. Used in the quantification of the yield of probe labeling	1 681 451

DIG DNA Labeling and Detection Kit 1093657

Vial	Description	Function	Cat. No. (if available separately)
1	one vial containing 20 µl **Unlabeled Control DNA 1** (100 µg/ml mixture of pBR328 DNA digested separately with *Eco* RI, *Bgl* I, and *Hin*f I. The separate digests are combined in a ratio of 2:3:3. Sizes [in basepairs] of the 16 pBR328 fragments are 4907, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154) in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0	used as a control target in a Southern Blot	
2	one vial containing 20 µl **Unlabeled Control DNA 2** (200 µg/ml pBR328 DNA that has been linearized with *Eco* RI)	used to practice labeling and to check labeling efficiency	
3	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+25°C])	used to prepare dilutions of the Labeled Control DNA (or experimental DNA)	
4	one vial containing 50 µl **Labeled Control DNA** (digoxigenin-labeled pBR328 DNA that has been random prime labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.)	used to estimate the yield of DIG-labeled DNA	1585738
5	one vial containing 50 µl **Hexanucleotide Mix** (10 x) (62.5 A_{260} units/ml random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithio-erythritol [DTE], 2 mg/ml BSA; pH 7.2)	contains hexamers and reaction buffer for the labeling reaction	1277081
6	one vial containing 50 µl **dNTP labeling mixture** (10 x) (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP, pH 6.5)	component of the labeling reaction	1277065
7	one vial containing 25 µl **Klenow enzyme, labeling grade** (2 units/µl DNA Polymerase I [Klenow enzyme, large fragment])	synthesizes DIG-labeled DNA	1008404
8	one vial containing 200 µl **Anti-Digoxigenin-AP** (750 units/ml polyclonal sheep anti-digoxigenin Fab fragments, conjugated to alkaline phosphatase)	binds to incorporated digoxigenin	1093274
9	two vials, each containing 1.25 ml **NBT** (75 mg/ml nitroblue tetrazolium salt in 70% [v/v] dimethyl-formamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383213 (100 mg/ml; dilute prior to use)
10	two vials, each containing 0.9 ml **BCIP** solution (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethyl-formamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383221
11	two bottles, each containing 50 g **Blocking Reagent** (Blocking reagent for nucleic acid hybridization; white powder)	blocks nonspecific binding of probes	1096176 (50 g)

DIG DNA Labeling Kit 1175033

Vial	Description	Function	Cat. No. (if available separately)
1	one vial containing 20 µl **Unlabeled Control DNA 1** (100 µg/ml mixture of pBR328 DNA digested separately with *Eco* RI, *Bgl* I, and *Hin*f I. The separate digests are combined in a ratio of 2:3:3. Sizes [in basepairs] of the 16 pBR328 fragments are 4907, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154) in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0	used as a control target in a Southern Blot	
2	one vial containing 20 µl **Unlabeled Control DNA 2** (200 µg/ml pBR328 DNA that has been linearized with *Eco* RI)	used to practice labeling and to check labeling efficiency	
3	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+ 25°C])	used to prepare dilutions of the Labeled Control DNA (or experimental DNA)	
4	one vial containing 50 µl **Labeled Control DNA** (digoxigenin-labeled pBR328 DNA that has been radom prime labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.)	used to estimate the yield of DIG-labeled DNA	1585738
5	one vial containing 80 µl **Hexanucleotide Mix** (10 x) (82.5 A_{260} units/ml random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithio-erythritol [DTE], 2 mg/ml BSA; pH 7.2)	contains hexamers and reaction buffer for the labeling reaction	1277081
6	one vial containing 80 µl **dNTP labeling mixture** (10 x) (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-dUTP, pH 6.5)	component of the labeling reaction	1277065
7	one vial containing 40 µl **Klenow enzyme, labeling grade** (2 units/µl DNA Polymerase I [Klenow enzyme, large fragment])	synthesizes DIG-labeled DNA	1008404

DIG Nucleic Acid Detection Kit 1175041

Vial	Description	Function	Cat. No. (if available separately)
1	one vial containing 50 µl **Labeled Control DNA** (digoxigenin-labeled pBR328 DNA that has been radom prime labeled according to the standard labeling procedure; the total DNA concentration in the vial is 20 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.)	used to estimate the yield of DIG-labeled DNA and used to practice detecting DIG-labeled DNA	1585738
2	one vial containing 1 ml **DNA dilution buffer** (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+ 25°C])	used to prepare dilutions of the Labeled Control DNA (or experimental DNA)	
3	one vial containing 200 µl **Anti-Digoxigenin-AP** (750 units/ml polyclonal sheep anti-digoxigenin, Fab fragments, conjugated to alkaline phosphatase)	binds to incorporated digoxigenin	1093274
4	two vials, each containing 1 ml **NBT** (75 mg/ml nitroblue tetrazolium salt in 70% [v/v] dimethylformamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383213 (100 mg/ml; dilute prior to use)
5	two vials, each containing 0.75 ml **BCIP solution** (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide)	precipitating substrate used to locate alkaline phosphatase-conjugated anti-DIG	1383221
6	two bottles, each containing 50 g **Blocking Reagent** (Blocking reagent for nucleic acid hybridization; white powder)	block nonspecific binding of probes	1096176 (50 g)

DIG RNA Labeling Kit 1175025

Vial	Description	Function	Cat. No. (if available separately)
1	one vial containing 40 µl pSPT18 DNA (0.25 mg/ml)	cloning/transcription vector; subclones are transcribed into RNA probes by T7 or SP6 RNA polymerase	
2	one vial containing 40 µl pSPT19 DNA (0.25 mg/ml)	cloning/transcription vector; subclones are transcribed into RNA probes by T7 or SP6 RNA polymerase	
3	one vial containing 20 µl Control DNA 1, pSPT18-Neo (0.25 mg/ml pSPT18-Neo DNA, cleaved with *Pvu* II)	used to practice making RNA probes with T7 RNA polymerase; results in DIG-labeled "antisense" Neo transcripts 760 bases in length	
4	one vial containing 20 µl Control DNA 2, pSPT19-Neo (0.25 mg/ml pSPT19-Neo DNA, cleaved with *Pvu* II)	used to practice making RNA probes with SP6 RNA polymerase; results in DIG-labeled "antisense" Neo transcripts 760 bases in length	
5	one vial containing 100 µl Labeled Control RNA (10 µg of digoxigenin-labeled "antisense" Neo RNA made with T7 RNA polymerase from 1 µg of Control DNA 1. Reaction products were phenol extracted, ethanol precipitated, and resuspended in 100 µl of DEPC-treated water. Template DNA is still present in the vial.)	used to estimate the yield of DIG-labeled RNA and used for hybridization with Unlabeled Control RNA (vial 6)	1585746
6	one vial containing 20 µl Unlabeled Control RNA (200 µg/ml unlabeled Neo poly(A) "sense" RNA, in DEPC-treated H_2O. The Neo poly(A) RNA is approximately 1 kb in length.)	target RNA used to practice RNA/RNA hybridizations; when applied to a membrane, this RNA will hybridize with the Labeled Control RNA (vial 5)	
7	one vial containing 40 µl NTP labeling mixture (10 x) (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-dUTP, in Tris-HCl, pH 7.5 (+ 20°C))	component of the labeling reaction	1277274
8	one vial containing 40 µl 10 x transcription buffer (400 mM Tris-HCl, pH 8.0; 60 mM $MgCl_2$; 100 mM dithioerythritol (DTE); 20 mM spermidine) 100 mM NaCl, 1 unit/ml RNase inhibitor))	component of the labeling reaction	
9	one vial containing 20 µl DNase I, RNase-free (10 units/µl)	degrades DNA template after the labeling reaction	776785
10	one vial containing 20 µl RNase inhibitor (20 units/µl)	prevents the degradation of RNA during the labeling reaction	799017
11	one vial containing 20 µl SP6 RNA Polymerase (20 units/µl)	synthesizes RNA from a DNA template	810274
12	one vial containing 20 µl T7 RNA Polymerase (20 units/µl)	synthesizes RNA from a DNA template	881767

DIG Oligonucleotide 3'-End Labeling Kit 1362 372

Vial	Description	Function	Cat. No. (if available separately)
1	one vial containing 100 µl 5 x reaction buffer (1 M potassium cacodylate*, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6 [+ 25°C])	optimized buffer for terminal transferase	
2	one vial containing 100 µl $CoCl_2$ solution (25 mM cobalt chloride)	cofactor required for optimal terminal transferase activity	
3	one vial containing 25 µl DIG-ddUTP (1 mM digoxigenin-11-ddUTP [2', 3' dideoxy-uridine-5'-triphosphate coupled to digoxigenin via an 11-atom spacer arm]) in redistilled water	digoxigenin-labeled nucleotide used for the addition of a single residue on to the 3' end of an oligonucleotide	1363 905
4	one vial containing 25 µl Terminal Transferase (50 units/µl, in 200 mM potassium cacodylate*, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50 % [v/v] glycerol; pH 6.5 [+ 25°C])	catalyzes addition of nucleotides to the 3' end of an oligonucleotide	220 582 (sold separately at 25 U/µl)
5	one vial containing 25 µl Control Oligonucleotide, Unlabeled (30-mer, 5'-p TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG OH-3', homologous to the *lac* Z' region in pUC and M13 plasmids), 20 pmol/ml, in redistilled water	used to practice labeling and to check labeling efficiency	
6	one vial containing 100 µl Control Oligonucleotide, DIG-ddUTP-labeled (2.5 pmol/µl; [sequence as in vial 5] labeled with DIG-ddUTP under standard kit assay reaction conditions) in redistilled water	used to estimate the yield of DIG-labeled oligonucleotide and used as a probe for Control DNA (Vial 7)	1585 754
7	one vial containing 20 µl Control DNA (0.25 mg/ml pUC18 DNA [supercoiled], in 10 mM Tris-HCl, 1 mM EDTA; pH 7.6 [+ 25° C])	used as a hybridization target for the Control Oligonucleotides (Vial 5 or 6)	885 797
8	one vial containing 50 µl Glycogen solution (20 mg/ml glycogen solution) in redistilled water	used as a carrier to increase the recovery of oligonucleotide after the labeling reaction	901 393
9	one vial containing 1ml DNA dilution buffer (50 µg/ml herring sperm DNA, in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 [+ 25°C]	used to prepare dilutions of the DIG-ddUTP-labeled Control Oligonucleotide (or experimental oligonucleotide)	

*Potassium cacodylate is toxic. Wear gloves when handling. Discard as regulated for toxic waste.

DIG Taq DNA Sequencing Kit for Standard and Cycle Sequencing** 1 449 443

Vial	Description	Function
1	one vial containing 40 µl **Control DNA, double-stranded** template plasmid DNA, pUC18 DNA with 40 µl pUC18 DNA, 0.25 µg/µl in Tris-EDTA buffer (TE buffer); pH 8	template for control sequencing reaction
2	one vial containing 25 µl **Control DNA, single-stranded** template DNA, M13mp18 DNA with 25 µl M13mp18 ssDNA, 0.2 µg/µl in TE buffer pH 8	template for control sequencing reaction
3	one vial containing 110 µl **M13/pUC sequencing primer**, 17-mer sequencing primer with the 5'-end labeled with digoxigenin 110 µl M13/pUC digoxigenin-labeled, 1 pmol/µl in water.	primer for sequencing reaction
4	one vial containing 110 µl **M13/pUC reverse sequencing primer**, 17-mer with the 5'-end labeled with digoxigenin reverse sequencing primer with 110 µl M13/pUC digoxigenin-labeled, 1 pmol/µl in water.	primer for sequencing reaction
5	one vial containing 250 µl **Reaction buffer**	buffer for the hybridization and chain elongation reaction
6	one vial containing 110 µl **Taq DNA Polymerase** DNA polymerase for the chain elongation reaction with 110 µl Taq DNA Polymerase, 3 units/µl.	component of the sequencing reaction
7	one vial containing 220 µl **Extension/termination mixture ddATP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddATP for termination reaction.	nucleotide mix for standard templates
8	one vial containing 220 µl **Extension/termination mixture ddCTP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddCTP for termination reaction.	nucleotide mix for standard templates
9	one vial containing 220 µl **Extension/termination mixture ddGTP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddGTP for termination reaction.	nucleotide mix for standard templates
10	one vial containing 220 µl **Extension/termination mixture ddTTP** (with dGTP, green), mixture containing dATP, dCTP, dGTP, dTTP, and ddTTP for termination reaction.	nucleotide mix for standard templates
11	one vial containing 220 µl **Extension/termination mixture ddATP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddATP for termination reaction.	nucleotide mix for GC-rich templates
12	one vial containing 220 µl **Extension/termination mixture ddCTP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddCTP for termination reaction.	nucleotide mix for GC-rich templates
13	one vial containing 220 µl **Extension/termination mixture ddGTP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddGTP for termination reaction.	nucleotide mix for GC-rich templates
14	one vial containing 220 µl **Extension/termination mixture ddTTP** (with 7-Deaza-dGTP, pink), mixture containing dATP, dCTP, 7-Deaza-dGTP, dTTP, and ddTTP for termination reaction.	nucleotide mix for GC-rich templates
15	one vial containing 1 ml **Formamide buffer solution**	stops the reaction

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Appendix B • Preparation of Additionally Required Solutions and Buffers

1. DNA/Southern Blotting and Hybridization Required Solutions and Buffers

Depurination solution
0.25 M HCl

Denaturation solution 1
(for Southern transfer and plaque hybridization):
0.5 N NaOH, 1.5 M NaCl

Denaturation solution 2
(for colony hybridization):
0.5 N NaOH, 1.5 M NaCl, 0.1% SDS

Neutralization solution 1
(for Southern transfer):
0.5 M Tris-HCl, pH 7.5
3 M NaCl

Neutralization solution 2
(for colony and plaque hybridization):
1.0 M Tris-HCl, pH 7.5
1.5 M NaCl

20 x SSC stock solution
3 M NaCl
0.3 M sodium citrate
pH 7.0 (+20°C), autoclaved

N-lauroylsarcosine stock solution
10% (w/v) in H_2O
filtered through a 0.2–0.45 µm membrane

SDS stock solution
10% (w/v) in H_2O
filtered through a 0.2–0.45 µm membrane

Blocking Reagent stock solution
Blocking Reagent is dissolved in maleic acid buffer to a final concentration of 10% (w/v) with shaking and heating. See Detection section for detailed instructions.

Standard hybridization buffer
5 x SSC
0.1% N-lauroylsarcosine
0.02% SDS
1% Blocking Reagent (from the 10% Blocking Reagent stock solution)

Standard hybridization buffer
+ 50% formamide
5 x SSC
50% formamide, deionized
0.1% sodium-lauroylsarcosine
0.02% SDS
2% Blocking Reagent.

High SDS concentration hybridization buffer
7% SDS
50% formamide, deionized*
5 x SSC
2% Blocking Reagent
50 mM sodium-phosphate, pH 7.0
0.1% N-lauroylsarcosine

For preparation of 500 ml of high SDS hyb buffers from stock solutions, combine the substances in the following order:

100% formamide, deionized*	250 ml
30 x SSC	83 ml
1 M sodium-phosphate, pH 7.0	25 ml
10% blocking solution	100 ml
10% N-lauroylsarcosine	5 ml

Pour the solution into an Erlenmeyer flask containing 35 g SDS (attention: wear respiratory protection). Heat the solution while stirring to dissolve the SDS, then fill up to 500 ml with autoclaved H_2O. The solution can be stored at 20°C and reused after heating to +65°C.

Probe stripping solution
(for alkali-labile dUTP)
0.2 N NaOH
0.1% SDS

2. RNA/Northern Blotting and Hybridization Required Solutions and Buffers

DMPC-treated H_2O
Dissolve dimethylpyrocarbonate (DMPC; Dimethyldicarbonate, e.g. Sigma D5520 or Velcorin®) to 1% in a 50% ethanol/water mixture. Mix redist. H_2O 1:10 with this solution (final concentration: 0.1% DMPC, incubate for 30 min at room temperature, then autoclave.
Note: diethylpyrocarbonate (DEPC) can also be used to treat solutions. We prefer DMPC because it is less toxic than DEPC.

RNA dilution buffer
Mix H_2O, 20 x SSC, and formaldehyde in the ratio of 5:3:2 respectively. The H_2O and 20 x SSC should have been treated with dimethylpyrocarbonate to destroy RNase activity.

RNA loading buffer
Make up a fresh solution.
250 µl formamide, deionized (see page 85)
83 µl formaldehyde 37% (w/v)
50 µl 10 x MOPS buffer
0.01% (w/v) bromophenol blue
50 µl glycerol
Fill up to 500 µl with DMPC-treated H_2O.

10 x MOPS
200 mM morpholinopropansulfonic acid
50 mM sodium acetate
10 mM EDTA
pH 7.0.
Make up in sterile H_2O or autoclave.
After autoclaving, the solution will turn yellow.

Hybridization buffers: see section 1.

Northern probe stripping solution
50% formamide
50 mM Tris-HCl, pH 8.0
1% (w/v) SDS.

or

DMPC-H_2O
0.1% SDS (w/v)

3. Detection Required Solutions and Buffers

Maleic acid buffer
0.1 M maleic acid
0.15 M NaCl
pH 7.5 (+20°C)
Adjust pH with concentrated or solid NaOH; autoclave.

Washing buffer
Add 0.3% (w/v) Tween® 20 to Maleic acid buffer.
Do not autoclave Washing buffer containing Tween® 20.

Maleic acid is available from Serva (Cat. No. 28337) and Sigma (Cat. No M 0375)

Blocking Reagent stock solution
Blocking Reagent is dissolved in Maleic acid buffer to final concentration of 10% (w/v) with stirring and heating either on a stir plate or in an microwave oven. The Blocking reagent must be heated while it dissolves in the Maleic acid buffer. Boiling will cause the reagent to coagulate, so care should be taken to AVOID BOILING during this step. This will be a turbid solution.

❶ Add 10 g Blocking Reagent to 100 ml Maleic acid buffer. Place on stir plate and heat to 60°C for approximately 1 h or until completely in solution. If necessary, the temperature may be raised to get the last of the blocking reagent into solution.

or

Dissolve 10 g Blocking Reagent in 100 ml Maleic acid buffer with several 30 s heat pulses in the microwave (3 to 4 min total).
Note: If Blocking Reagent doesn't go into solution, check pH of solution, adjust if necessary, and reapply to heat.

❷ If necessary, treat with 0.1% DMPC (dimethylpyrocarbonate) to destroy RNases.

❸ Autoclave the solution using a regular program, such as that used for the sterilization of cell culture medium.
Note: Blocking reagent must be completely in solution before autoclaving.

❹ Store autoclaved solution at room temperature (unopened), +4°C or 20°C.

❺ Check before each use for contamination.

Blocking buffer
Dilute Blocking Reagent stock solution 1:10 with Maleic acid buffer.

Detection buffer
100 mM Tris-HCl, pH 9.5 (+20°C)
100 mM NaCl

TE buffer
10 mM Tris-HCl
1 mM EDTA
pH 8.0 (+20°C)

Color substrate solution
(freshly prepared)
45 µl NBT solution and 35 µl BCIP solution are added to 10 ml Detection buffer.

4. General Solutions and Buffers

20 x SSC
3 M NaCl
300 mM sodium citrate, pH 7.0

Washing solution 2 x
2 x SSC
0.1% SDS

Washing solution 0.5 x
0.5 x SSC
0.1% SDS

Washing solution 0.1 x
0.1 x SSC
0.1% SDS

N-lauroylsarcosine
10% (w/v) in sterile H_2O
filtered through a 0.2–0.45 µm membrane

SDS
10% (w/v) in sterile H_2O
filtered through a 0.2–0.45 µm membrane

Formamide
Deionization of formamide
50 g ion exchange: AG 501-X8 Resin (Bio-rad)
500 ml formamide
Stir 30 min slowly on a stirrer, then remove resin by filtration and store the deionized formamide at 20°C.

Appendix C • DIG System Product Listing

DIG Nonradioactive Nucleic Acid Labeling and Detection System

DNA Probe Labeling

Product	Cat. No.	Size
DIG High Prime Labeling and Detection Starter Kit I (for color detection with NBT/BCIP)	1745 832	12 labeling reactions and 24 blots (10 x 10 cm)
DIG High Prime Labeling and Detection Starter Kit II (for chemiluminescent detection with CSPD, ready to use)	1585 614	12 labeling reactions and 24 blots (10 x 10 cm)
DIG High Prime	1585 606	160 µl (40 reactions)
DIG DNA Labeling and Detection Kit	1093 657	25 labeling reactions and 50 blots (10 x 10 cm)
DIG DNA Labeling Kit	1175 033	40 labeling reactions
DIG DNA Labeling Mixture	1277 065	50 µl (25 reactions)
DIG-Nick Translation Mix	1745 816	160 µl (40 reactions)
PCR DIG Probe Synthesis Kit	1636 090	25 reactions
Digoxigenin-11-dUTP, alkali-labile	1573 152 1573 179	25 nmol (25 µl) 125 nmol (125 µl)
Digoxigenin-11-dUTP, alkali-stable	1093 088 1558 706 1570 013	25 nmol (25 µl) 125 nmol (125 µl) 5 x 125 nmol (5 x 125 µl)
DIG-labeled control DNA	1585 738	50 µl
DIG Quantification Teststrips	1669 958	50 strips
DIG Control Teststrips	1669 966	25 strips

Oligonucleotide Probe Labeling

Product	Cat. No.	Size
DIG Oligonucleotide 3'-End Labeling Kit	1362 372	25 reactions
DIG Oligonucleotide Tailing Kit	1417 231	25 reactions
DIG Oligonucleotide 5'-End Labeling Set	1480 863	10 reactions
Digoxigenin-11-dUTP, alkali-labile	1573 152 1573 179	25 nmol (25 µl) 125 nmol (125 µl)
Digoxigenin-11-dUTP, alkali-stable	1093 088 1558 706 1570 013	25 nmol (25 µl) 125 nmol (125 µl) 5 x 125 nmol (5 x 125 µl)
Digoxigenin-11-ddUTP	1363 905	25 nmol (25 µl)
Aminolinker	1685 643	100 mg
Digoxigenin-3-O-methyl-carbonyl-ε-amino-caproic acid-N-hydroxysuccinimide ester (DIG-NHS-ester)	1333 054	5 mg
DIG-3'-end labeled control oligonucleotide	1585 754	50 µl (125 pmol)
AP-Oligonucleotide Labeling Kit	1745 859	10 reactions (for 20 nmol oligonucleotide each)

RNA Probe Labeling

Product	Cat. No.	Size
DIG RNA Labeling Kit (SP6/T7)	1175025	2 x 10 reactions
DIG RNA Labeling Mix	1277073	40 µl (20 reactions)
Digoxigenin-11-UTP	1209256	250 nmol (25 µl)
DIG-labeled control RNA	1585746	50 µl
DIG Quantification Teststrips	1669958	50 strips
DIG Control Teststrips	1669966	25 strips

Hybridization

Product	Cat. No.	Size
Nylon Membranes, positively charged	1209272 1209299 1417240	10 sheets (20 x 30 cm) 20 sheets (10 x 15 cm) 1 roll (0.3 x 3 m)
Nylon Membranes for Colony and Plaque Hybridization	1699075 1699083	50 filters (Ø 82 mm) 50 filters (Ø 132 mm)
DIG Easy Hyb	1603558	500 ml
Blocking Reagent	1096176	50 g
Hybridization Bags	1666649	50 bags

Digoxigenin Detection

Product	Cat. No.	Size
DIG High Prime Labeling and Detection Starter Kit I (for color detection with NBT/BCIP)	1745832	12 labeling reactions and 24 blots (10 x 10 cm)
DIG High Prime Labeling and Detection Starter Kit II (for chemiluminescent detection with CSPD, ready to use)	1585614	12 labeling reactions and 24 blots (10 x 10 cm)
DIG Luminescent Detection Kit	1363514	50 blots (10 x 10 cm)
DIG Nucleic Acid Detection Kit	1175041	40 blots (10 x 10 cm)
Anti-Digoxigenin-AP, Fab Fragments	1093274	150 U (200 µl)
Multicolor Detection Set	1465341	1 set (3 x 50 tablets)
CDP-Star™	1685627 1759051	1 ml 2 ml
CSPD®	1655884 1759035 1759043	1 ml 2 ml 4 ml
CSPD®, ready to use	1755833	2 x 50 ml
NBT/BCIP Ready-to-use Tablets	1697471	20 tablets
NBT/BCIP Stock Solution	1681451	8 ml
5-Bromo-4-chloro-2-indolyl-phosphate (BCIP)	1383221	3 ml (150 mg)
4-Nitro Blue Tetrazonium chloride (NBT)	1383213	3 ml (300 mg)
Fast Red Tablets	1496549	20 tablets
HNPP Fluorescent Detection Set	1758888	5 mg HNPP; 100 mg Fast Red
DIG Wash and Block Buffer Set	1585762	30 blots (10 x 10 cm)
Blocking Reagent	1096176	50 g
Lumi-Film, for Chemiluminescent Detection	1666657 1666916 1666711	100 sheets (8 x 10 cm) 100 sheets (18 x 24 cm) 100 sheets (35 x 43 cm)

Nonradioactive Sequencing

Product	Cat. No.	Size
DIG Taq DNA Sequencing Kit	1 449 443	1 kit (100 reactions)
Long Range Termination Set for Taq Sequencing	1 749 838	1 set (100 reactions)
Lambda gt11 sequencing primer, 5'-digoxigenin labeled	1 573 225	100 pmol
Lambda gt11 reverse sequencing primer, 5'-digoxigenin labeled	1 573 233	100 pmol
M13/pUC sequencing primer, 5'-digoxigenin labeled	1 544 497	100 pmol
M13/pUC reverse sequencing primer, 5'-digoxigenin labeled	1 544 519	100 pmol
SP6 promotor specific primer, 5'-digoxigenin labeled	1 573 195	100 pmol
T3 promotor specific primer, 5'-digoxigenin labeled	1 573 209	100 pmol
T7 promotor specific primer, 5'-digoxigenin labeled	1 573 217	100 pmol
Digoxigenin-16-dATP	1 558 714	2.5 nmol (25 µl)
Fluorescein-15-dATP	1 498 142	25 nmol (25 µl)
DIG Oligonucleotide 5'-End Labeling Set	1 480 863	1 set (10 reactions)
Luminescent Detection Set for DIG Sequencing	1 733 915	1 set (10,000 cm² membrane)

Fluorescein Labeling and Detection

Product	Cat. No.	Size
Fluorescein-High Prime	1 585 622	100 µl (25 reactions)
PCR-Fluorescein Labeling Mix	1 636 154	100 µl (10 reactions)
Nick Translation Mix	1 745 808	200 µl (50 reactions)
Fluorescein-12-dUTP	1 373 242	25 nmol (25 µl)
Fluorescein RNA Labeling Mix	1 685 619	40 µl (20 reactions)
Fluorescein-12-UTP	1 427 857	250 nmol (25 µl)
Fluorescein-12-ddUTP	1 427 849	25 nmol (25 µl)
Anti-Fluorescein-AP, Fab fragments	1 426 338	150 U (200 µl)
Anti-Fluorescein-POD, Fab fragments	1 426 346	150 U

Biotin Labeling and Detection

Product	Cat. No.	Size
Biotin-High Prime	1 585 649	100 µl (25 reactions)
Biotin-Nick Translation Mix	1 745 824	160 µl (40 reactions)
Biotin-16-dUTP	1 093 070	50 nmol (50 µl)
Biotin RNA Labeling Mix	1 685 597	40 µl (20 reactions)
Biotin-16-UTP	1 388 908	250 nmol (25 µl)
Biotin-16-ddUTP	1 427 598	25 nmol (25 µl)
Anti-Biotin-AP, Fab fragments	1 426 303	150 U (200 µl)
Anti-Biotin-POD, Fab fragments	1 426 311	150 U
Streptavidin-AP, for nucleic acid detection	1 093 266	150 U
Streptavidin-POD	1 098 153	500 U

Molecular Weight Marker, digoxigenin-labeled

Product	Cat. No.	Size
DNA Molecular Weight Marker II, digoxigenin-labeled	1218590	5 µg (500 µl)
DNA Molecular Weight Marker III, digoxigenin-labeled	1218603	5 µg (500 µl)
DNA Molecular Weight Marker V, digoxigenin-labeled	1669931	5 µg (500 µl)
DNA Molecular Weight Marker VI, digoxigenin-labeled	1218611	5 µg (500 µl)
DNA Molecular Weight Marker VII, digoxigenin-labeled	1669940	5 µg (500 µl)
DNA Molecular Weight Marker VIII, digoxigenin-labeled	1449451	5 µg (500 µl)
RNA Molecular Weight Marker I, digoxigenin-labeled	1526529	4 µg (200 µl)
RNA Molecular Weight Marker II, digoxigenin-labeled	1526537	2 µg (200 µl)
RNA Molecular Weight Marker III, digoxigenin-labeled	1373099	2 µg (200 µl)

Other DIG System Reagents

Product	Cat. No.	Size
DIG Gel Shift Kit	1635352	1 kit
Biotin *In Vitro* Translation Kit	1559451	1 kit (30 translations)
Anti-Digoxigenin-POD, Fab fragments	1207733	150 U
Anti-Digoxigenin-POD (poly), Fab fragments	1633716	50 U
Actin RNA Probe, digoxigenin-labeled	1498045	2 µg
DNA probe, specific for all *S. cerevisiae* chromosomes, digoxigenin-labeled	1573187	500 ng (100 µl)
Magnetic Particle Separator	1641794	1 Separator
Anti-DIG Magnetic Particles	1641751 1641760	20 mg (2 ml) 100 mg (10 ml)

Boehringer Mannheim offers additional reagents, intended for use in *in situ* hybridization, like

- fluorescent labeled nucleotides
- fluorescent labeled antibodies
- fluorescein-/digoxigenin-labeled chromosome specific probes
- enzyme substrates

For a complete overview please refer to the Biochemicals Catalog or to the "Nonradioactive *In Situ* Hybridization Application Manual".

Appendix D • Trouble-Shooting Guide

Trouble Shooting and General Hints on Good Laboratory Practice for DIG Labeling and Detection

Here we describe trouble shooting and general suggestions for good laboratory practice with the DIG System.

Work under sterile conditions
- ▶ Autoclave DIG System solutions.
- ▶ Filter-sterilize solutions containing SDS; Tween® 20 should be added to previously sterilized solutions.
- ▶ Use sterile pipette tips.
- ▶ For preparation of solutions, see Appendix B.

Use clean incubation trays
- ▶ Rigorously clean and rinse laboratory trays before each use.
- ▶ When Northern Blots are performed, use the sterile glass trays for all washing and detection steps.

Membrane handling requirements
- ▶ Wear powder-free gloves when handling membranes.
- ▶ Handle membrane only on the edges and with clean forceps.

Important Hints for Handling

Below, handling is described for all important steps, and the influence on sensitivity and background with the DIG System is indicated.

1. Labeling Reactions	Influence on background	Influence on sensitivity
1.1. Incorporation of Digoxigenin-11-dUTP during PCR Amplified vector sequences can lead to non-specific hybridization signals. Be sure to exclude vector sequences from the labeling reaction: digest the vector with a restriction enzyme such that its recognition site is as close as possible to the primer-binding sites.	+	
By-products of the PCR results in non-specific hybridization. We recommend to purify the specific band on agarose gels after labeling. When high amounts of by-products are formed, decrease the amount of template.	+	
1.2. Random primed labeling Most important step: denaturing the probe. Be sure to denature the probe; boil for 10 min at 100°C (use a waterbath with lid), and place it immediately on ice.		+
Do not label vector sequences. Use only fragments ≤ 10 kb; digest longer probes with a 4 bp cutting restriction enzyme. The smallest fragment tested so far in random primed labeling was 52 bp; the sensitivity of the reaction dropped to 1.0 pg.		+
Templates should be purified with phenol chloroform extractions prior to labeling. If a probe does not reveal a reasonable sensitivity in the direct detection assay, repurify via Elu-Tip® minicolumns (Schleicher & Schuell). This is especially necessary after fragment isolation from agarose. DNA fragments can be directly labeled after agarose treatment without further purification. After isolation with the Agarose Gel DNA Extraction Kit, labeling can also be performed without problems.		+
The labeling reaction can be upscaled. This results in a higher yield of probe. A higher efficiency can also be obtained by overnight incubation.		+
Proteinase K treatment of the completed labeling reaction can enhance the sensitivity and reduce background: resuspend the labeled probe after ethanol precipitation in 50 µl sterile H_2O, add 1/10 volume of Proteinase K (20 mg/ml) and incubate for 2 h at +37°C. The mixture can directly be used for hybridization.	+	+
Recent experiments have shown that it is not absolutely necessary to separate the unincorporated DIG-11-dUTP nucleotides from the labeled probes.		

1.3. 3'-End labeling/tailing Make sure that the oligonucleotide concentration has been determined correctly. We calculate 1 OD_{260} = 33 µg oligonucleotide. The concentration of short oligonucleotides of known sequence can also be determined using A_{260}. However, the base composition of the oligonucleotide can have significant effects on absorbance. The total absorbance is the sum of the individual contributions of each base.		+
When upscaling the labeling reaction, all components have to be increased proportionally. Increasing only the oligonucleotide concentration results in insufficient labeling.		+
Sometimes it is difficult to precipitate short, labeled oligonucleotides quantitatively. Make sure that all solutions (LiCl, Glycogen dilution, ice-cold ethanol) are mixed thoroughly with the reaction mix. Instead of the Glycogen dilution, 1 µl of concentrated Glycogen can be used (see page 39).		+
1.4. RNA labeling by *in vitro* transcription Use sterile disposable plastic ware and sterile DMPC-treated solutions.		+
Linearize template DNA for *in vitro* transcription to receive a vector-free probe and better labeling efficiency.		
Be sure to use the correct strand for RNA transcription if RNA on a Northern Blot is hybridized. Transcripts can also be analyzed on nondenaturating agarose gels using TAE buffer.	+	+
For all labeling reactions it is extremely important that you check the labeling efficiency in a direct detection assay prior to hybridization.		++

2. Blotting	**Influence on background**	**sensitivity**
Various methods are available for the transfer of nucleic acids from agarose gels to membranes. Most commonly used methods in order to achieve high sensitivity: capillary blot > vacuum blot > dry blot.		+
2.1. Pretreatment of DNA in the agarose gel prior to transfer Depending on the size and structure of the DNA, pretreatment of the gel may be necessary for optimal transfer.		+
DNA fragments larger than 10 kb and supercoiled plasmids have to be fragmented inside the gel after electrophoresis. This can either be achieved by treating the gel with 0.25 M HCl for 5–20 min (start with 5–10 min for mammalian DNA; this can be prolonged up to 20 min, especially for plant DNA) or by UV-irradiation on a transilluminator. The latter has the advantage that only those parts of the gel containing long fragments can be exposed to UV light so that smaller fragments remain unaffected.		+
Note: For every transilluminator, irradiation conditions have to be defined empirically in a test series. With HCl treatment, be sure not to over degrade small fragments, causing them to be lost during transfer.		+
For the transfer of Mb-size fragments and supercoiled plasmids, a combination of both methods may be necessary. Conditions also have to be defined empirically.		+
2.2. Denaturation Unless alkaline transfer is performed, double-stranded DNA has to be denatured in the gel prior to transfer. This can be accomplished with 0.5 M NaOH; 1.5 M NaCl (for gels and membranes; e.g., Colony and plaque hybridization).		+
Subsequent neutralization can be enhanced by briefly washing the gel in distilled water after alkaline treatment.		
2.3. Neutralization Especially when transfer to nitrocellulose membranes is intended, it is important to check the actual pH of the gel after neutralization. It should be below pH 9 (nylon membranes will tolerate a higher pH); otherwise, membranes will turn yellow and break during hybridization. To check the pH of the gel, lift one edge of the gel where no DNA has been loaded, press a pH stick onto it, and read the pH.		

2.4. Setting up a capillary transfer Nylon membranes need no pretreatment. Nitrocellulose membranes have to be pre-wetted in distilled water and 20 x SSC prior to transfer. We strongly recommend the use of the Boehringer Mannheim Nylon Membranes, which are especially designed and function tested for DIG System blotting applications. Membranes have to be handled extremely carefully. Pick up membranes only with forceps at the corners; wear powder-free gloves; be careful not to leave any fingerprints.	+	+
It is important to remove air bubbles between the gel and the membrane. This can be achieved by rolling a clean pipette gently across the membrane.		+
Place Parafilm® around the gel to avoid contact of the blotting paper with the Whatman paper and the transfer buffer underneath the gel.		+
Use 20 x SSC for the transfer; 10 x SSC can only be applied if very large DNA fragments are transferred. Capillary blots should be performed overnight.		+
Make sure that the transfer buffer is soaked only through the gel and not around the gel. Place the paper towels so that no contact between Whatman paper underneath the gel or transfer buffer is possible. Replace the blotting paper when it becomes wet.		+
The weight applied should be in accordance with the gel size. We suggest 500 g for mini gels and a maximum of 1250 g (20 x 20 cm gels).		+
After UV fixation, carefully wash the membrane quickly in 2 x SSC to avoid salt precipitates.	+	
Stain the gel with ethidium bromide to evaluate the efficiency of the transfer.		+
2.5. Fixation The DNA has to be fixed to the membrane prior to hybridization. This can be achieved either by UV-crosslinking (with a Stratalinker® or transilluminator) or by baking.	+	+
For most membranes, baking at +80°C for 2 h is sufficient. The Boehringer Mannheim positively charged Nylon Membrane should be baked at +120°C, but for no more than 30 min. In general, UV-crosslinking will probably be more convenient.	+	+
Especially if chemiluminescent detection is to be performed, it is important to UV-crosslink the membrane from both sides. This reduces the background coming from the opposite side of the membrane. When light is emitted during signals from both sides of the membrane will reach the X-ray film.	+	
Special hints for Northern Blots: pre-soak gels in sterile transfer buffer for 20 min prior to transfer. All types of RNA gel systems are compatible with the DIG System.		+
Ethidium bromide staining of RNA gels does not interfere with transfer. Autoclave the transfer buffer. When handling the membrane, it must be taken into account that RNases can degrade single-stranded RNA bound to a membrane.		+
3. Hybridization	**Influence on background**	**sensitivity**
3.1. Prehybridization DIG Easy Hyb is the preferred buffer for all applications.	+	+
Working concentrations for Blocking Reagent during prehybridization are between 1–5%. Use a sterile Blocking Reagent stock solution (10%; see page 83 for a description of its preparation) to prepare the prehybridization solution. Denatured fish sperm DNA or yeast RNA at a final concentration of 50 µg/ml can be added but is not necessary for most applications.	+	
Take into account that hybridization signals could eventually be blocked by cross hybridization of the target DNA with a nonspecific nucleic acid.		+
Allow the prehybridization to proceed for at least 1 h at the same temperature as the subsequent hybridization. Use a sufficient volume of prehybridization solution. If roller tubes are applied, use a minimum of 20 ml. If sealed bags or trays are used, gently shake them during the prehybridization.	+	
Do not allow the membranes to dry between prehybridization and hybridization.	++	

3.2. Hybridization with probe **Note: it is important to carefully evaluate the correct hybridization conditions for a given probe and target.**	+	+
Probe concentration is a very important factor. A probe concentration that is too high may lead to non-specific binding of the probe to the membrane, and a probe concentration that is too low could lead to lower sensitivity. The concentrations given in this guide will work with most routine applications.	+	+
Shorter hybridization times can be applied in combination with a higher probe concentration. For important experiments, we recommend that you test the optimal probe concentration in a mock hybridization. For this purpose, pre-incubate small pieces of membrane, and then hybridize them overnight with increasing concentrations of labeled probe per ml of hybridization solution. After detection, the optimal concentration can be defined (see page 42 for detailed description).	+	+
When CDP-Star™ is used for chemiluminescent detection, the optimal probe concentration is typically half the concentration that is used for other types of detection. As starting point we recommend 10–20 ng/ml DIG-labeled DNA or 20–50 ng/ml DIG-labeled RNA.	+	+
3.2.1. Denaturation of probe Denature DNA probes and RNA probes (secondary structures) before adding them to the hybridization solution.		+
With oligomers, denaturation is only necessary when secondary structures can be expected from the nucleotide sequence.		
Use only deionized formamide (if it is included in the hybridization solution).	+	+
3.2.2. Hybridization in roller tubes Use at least 6 ml per hybridization solution tube. This volume can be increased if required.	+	+
Monitor the hybridization temperature. Note that the temperature set on the oven is not necessarily the temperature maintained inside the roller tube. Check the temperature inside the tube before hybridization by filling the tube, for instance, with water and placing a thermometer inside the tube.	+	+
3.2.3. Hybridization in sealed bags Use at least 3.5 ml hybridization solution per 100 cm^2 of membrane. This volume can be increased (e.g. to 5 ml) when sufficient amounts of probe are available.	+	+
Remove all air bubbles prior to sealing.	+	+
Check the seals.		
Gently shake sealed bags in a waterbath set at the right hybridization temperature. The membrane should lie flat on the bottom of the waterbath. Uneven positioning of the membrane will cause loss of sensitivity and background problems. Membranes can also be placed flat in an incubator.	+	+
3.2.4. Special hints for hybridization with tailed oligonucleotides Hybridization with a tailed oligonucleotide should be performed with 0.1 µg/ml Poly (A) in the prehybridization and hybridization solution to prevent non-specific hybridization signals. Additionally, 5 µg/ml Poly d(A) can increase blocking efficiency.		+

3.3. Special hints for Northern Blot applications The preferred hybridization buffer for Northern Blots is DIG Easy Hyb. Alternatively you may use the High SDS buffer (see page 83). Buffers without 50% formamide should not be used (except DIG Easy Hyb).	+	+
It is preferable to work with RNA probes whenever possible.		+
Work under sterile conditions.	+	+
Single-stranded RNA can be degraded by single-strand-specific RNases also when bound to membrane, and double-stranded RNA hybrids can be degraded by double-strand-specific RNases.		+
Special hint for Northern Blots when RNA probes have been used: background can be reduced by adding an RNase A wash step after the last stringent wash. Use a 100 µg/ml RNase A solution in 10 mM Tris-buffer, 5 mM EDTA, 300 mM NaCl, pH 7.5 for 30–60 min at room temperature. **Attention:** some RNase A preparations may contain double-strand-specific RNases that can degrade hybrids on the membrane. Double-strand-specific RNases can be detected by incubation with double stranded Poly RNAs or MS2 RNA followed by analysis on a denaturating agarose gel. MS2 RNA is partially double-stranded. RNase A preparations that do not contain double-strand-specific RNase activity will leave these regions unaffected. It is always necessary to compare the untreated MS2 RNA when performing such tests.	+	+
3.4. Washes Optimize the washing conditions for your particular application. The conditions given in the pack insert are defined for 100% homology between target DNA and probe, and a GC content of about 50%. For genomic hybridizations, we recommend the use of 0.5 x SSC for the stringency washes. However, it may be necessary to increase the stringency (e.g. 0.1 x SSC).	+	+
Apply vigorous shaking during the stringency washes.	+	
Prewarm the wash solutions to the appropriate temperature.	+	
Use trays rather than roller tubes for the washing steps.	+	
Do not allow the membranes to overlap or stick together during the washing steps.	+	
Use an excess volume of washing solution.	+	
4. Immunological Detection	**Influence on background**	**sensitivity**
Use freshly washed trays.	+	
Shake membranes during the whole detection procedure (except color development).	+	
Store the antibody at + 4° C. Carefully check the tube to see if a precipitate has formed. If so, remove the precipitate by a 30 sec centrifugation.	+	
Work under sterile conditions.	+	
The blocking and washing steps can be prolonged, but do not prolong the antibody reaction.	+	
The concentration of the Blocking Reagent can be increased to up to 5%.	+	
Use freshly washed trays after the antibody reaction.	+	
Prepare a fresh dilution of antibody and color substrate solutions directly before use.	+	
When working with the chemiluminescent substrates, a dilution of only 1:10,000 (CDP-Star™ 1:20,000) of the antibody is necessary.	+	
Work under absolute sterile conditions when handling the chemiluminescent substrate solution, and avoid phosphatase contamination.	+	
Allow the color reaction to develop in the dark without shaking. It is not necessary to work in the dark with CSPD® or CDP-Star™.	+	
It is possible to switch from chemiluminescent detection to a color reaction on the same blot. Wash off the chemiluminescent substrate for 5 min with Detection buffer, and then add the color substrate. Background from the opposite side of the membrane is then excluded. It is not a problem if the higher antibody dilution has been applied previously.	+	–

Dilutions of CSPD® and CDP-Star™ can be reused one or two times within a month. Avoid contamination and store in the dark at +4°C.	+	
Films with different sensitivities are available. Use Lumi-Film for best results.		+

5. Special Hints for Colony and Plaque Hybridization	Influence on background	Influence on sensitivity
It is very important that all cellular debris is removed before proceeding to the hybridization. Follow the protocol on page 50.	+	
It is extremely important that no vector sequences are present in the probe preparation.	+	

6. Application of Other Hybridization Buffers with the DIG System	Influence on background	Influence on sensitivity
All established hybridization protocols can be used with the DIG System. Buffers given in this User's Guide have been specially optimized to work with the DIG System. Denhardt's solution can be used instead of Blocking Reagent. The bands obtained are slightly fuzzy compared to those obtained with Blocking Reagent.	+	+

7. Signal Intensification	Influence on background	Influence on sensitivity
Hybridization signals can be intensified by the addition of 10% dextran sulphate or 6% PEG 8000, but the background will also increase. Dextran sulphate shows lot-to-lot inconsistency, with some lots, a strong background is obtained. PEG is more reliable in this respect, but we have not observed any increase in sensitivity. Only the signal intensity itself could be increased 2-3-fold.	+	+

8. Stripping and Reprobing		
In addition to the methods given in the pack inserts, stripping by boiling in distilled water containing 0.1% SDS can be recommended. This is especially useful when stripping northern blots because alkali treatment degrades the RNA on the blot as well as in cases where digoxigenin-labeled molecular weight markers have been transferred to the membrane.		

9. Molecular Weight Markers		
It is very convenient to use DIG-labeled molecular weight markers. The markers are visualized automatically during the detection reaction, simplifying the calculation of the molecular weight of bands of interest. **Note: DIG-labeled molecular weight markers do not withstand alkali transfer and alkaline stripping.**		

To become familiar with the DIG System, start the control reactions given in the kit.

Problem 5: (a) inadequate sensitivity or (b) high background

A possible cause:
(a) underexposure
(a) overexposure

Note:
For further reasons that might cause the same problem, please refer to pages 90–95.

Recommendation:
Adjust the exposure time.

Problem 6: high background

A possible cause:
The probe concentration was too high, or the template DNA was contaminated.

Note:
For further reasons that might cause the same problem, please refer to pages 90–95.

Recommendation:
Perform a mock hybridization as described on page 42 to determine the highest probe concentration that can be used without resulting in high background.

Prolong the stringency wash steps (2 x 20 min) and the antibody wash steps (2 x 20 min).

Problem 7: Irregular smears of background

A possible cause:
Non-uniform distribution of chemiluminescent substrate during chemiluminescent detection; certain parts of the membrane are dry.

Note:
For further reasons that might cause the same problem, please refer to pages 90–95.

Recommendation:
Refer to the detection procedure on page 58 where two methods for the distribution of the chemiluminescent substrate are described.

Irregular smears of background can also be caused by a crumpled hybridization bag. The bag crumples because of the heat, and this crumples the membrane in the same pattern so that the X-ray film does not have uniform contact with the membrane. To avoid this problem, make sure that the surfaces of the bag are smooth before hybridization is initiated.

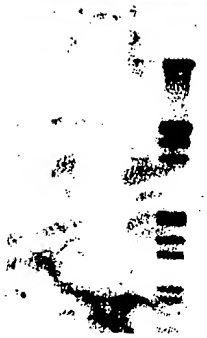
Problem 8: spots on the exposed X-ray film outside the area where the membrane had contact

A possible cause:
The outside spots on the exposed X-ray film are caused by electrostatic charge on the sealing bag.

Note:
For further reasons that might cause the same problem, please refer to pages 90–95.

Recommendation:
Wipe the surface of the sealing bag with 70% ethanol before applying the film.

Wear gloves and touch the membranes only at the edges with a clean forceps.



Problem 9: clouds of background on the autoradiograph

A possible cause:
Distribution of probe was uneven.

Note:
For further reasons that might cause the same problem, please refer to pages 90–95.

Recommendation:

- ▶ Use at least 3.5 ml hybridization solution per 100 m^2 membrane.
- ▶ Shake during hybridization, and make sure the bag lies flat on the bottom of the water bath.
- ▶ If a roller apparatus is used, apply at least 6 ml hybridization solution per tube.
- ▶ Do not allow the membrane to dry between prehybridization and hybridization.

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